Binding characteristics, G-protein activation and receptor regulatory mechanisms of opioid ligands

Ph.D. thesis

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Szeged, 2000

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **B. Bozó**, F. Fülöp, Gábor Tóth, Géza Tóth, M. Szűcs (1997) Synthesis and opioid binding activity of dermorphin analogues containing β-amino-cyclic carbonic acids. *Neuropeptides* **31:** 367-372.

II. **B. Bozó,** J. Farkas, G. Tóth, M. Wollemann, M. Szűcs and S. Benyhe (2000) Receptor binding and G-protein activation by new Met^5-enkephalin-Arg^6-Phe^7 derived peptides *Life Sci.* **66:** 1241-1251.

III. M. Szűcs, **B. Bozó**, E. G. Kicsi, G. Fábián, Gy. Horváth, M. Szikszay, M. Mácsai, Gy. Szabó Distinct Receptor Regulatory Mechanisms are Induced by Individual Opioid Agonists in Rat Brain *J. Neurochem* (submitted)

IV. G. Fábián, Cs. A. Szabó, **B. Bozó**, M. Szűcs, M. A. Deli, I. A. Krizbai and F. Joó (1998) Expression of G-protein subtypes in cultured cerebral endothelial cells. *Neurochem. Intern.* **33:** 179-185.

CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Mária Szűcs, for giving me the opportunity to perform this work in her laboratory. I am also grateful for her help, valuable criticism and support.

The opportunity is thanked to the Neuroendocrinology Ph.D. program of the SZAOTE, for providing a 3-year fellowship.

I am thankful to my consultant, Dr. Botond Penke for his support during these years.

Many thanks for the help of my colleagues, Gabriella Fábián and Emese Kicsi.

I am also grateful to Ildikó Németh for her excellent technical assistance and to Marika Tóth for the preparation of slides and figures.

Thanks to Dr. Mária Wollemann, Dr. Anna Borsodi and all her colleagues for the advice, suggestions and assistance.

I am grateful to Dr. Géza Tóth and his coworkers for synthesizing the ligands and radioligands for this study.

I would like to thank Dr. Margit Szikszay and Dr. Gyula Szabó for their help in treating animals.

My warmest thanks to my family for the patience, love and support.

ABBREVIATIONS

B_{max}	maximal number of binding sites
DADLE	[D-Ala^2,Leu^5]enkephalin
DAMGO	Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol
DAMCK	Tyr-D-Ala-Gly-(NMe)Phe-CH_2Cl
DSLET	[D-Ser^2,Leu^5,Thr^6]enkephalin
DTT	dithio-threitol
EC_{50}	concentration producing 50% stimulation
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EKC	ethylketocyclazocine
G-protein	guanine nucleotide binding protein
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine-5'-O-(3-thio)triphosphate
IC_{50}	concentration producing 50% inhibition
ICI174,864	N,N-diallyl-Tyr-Aib-Aib-Phe-Thr (Aib = aminoizobutylic acid)
ICI197,067	(2S)-N-[2-(N-methyl-3,4-dichlorophentanylacetamid)-3-methylbutyl]pirrolidin hydroxychloride
k_{+1}	assotiation rate constant
k_{-1}	dissotiation rate constant
K_d	equilibrium dissociation constant
K_i	inhibition constant
LM	light vesicular fraction
nor-BNI	norbinaltorphimine
PKA	protein kinase A
PKC	protein kinase C
PLO17	Tyr-Pro-MePhe-D-Pro-NH_2
POMC	proopiomelanocortin
SEM	standard error of the mean
SPM	synaptic plasmamembranes
TIPP	Tyr-Tic-Phe-Phe-NH_2
Tris	tris-(hydroxymethyl)-aminomethane
U-69,593	5α,7α,8β-(-)-N-methyl-N[7-(1-pyrrolidinyl)-1oxaspiro(4-5)dec-8-yl]benzyle-acetamide
U-50,488	trans-3,4-dichloro-N-(methyl)-N-[2-(1- pyrrolidinyl)-cyclohexyl]-benzene-acetamide

SUMMARY

The extracellular signals of opioid ligands are mediated by opioid receptors, that are located on the opiate sensitive neurones in the central nervous system, consist of seven transmembrane segments and interact with heterotrimeric guanine nucleotide binding proteins (G-proteins). This thesis deals with the receptor binding properties, G-protein activation and receptor regulatory effects of naturally occurring and synthetic ligands that interact with opioid receptors.

In the firs part of the study 8 conformationally constrained analogues of the μ–specific opioid peptide dermorphin, which were synthesized by replacing D-Ala^2 with stereoisomers of β-amino-cycloalkane or cycloalkene carboxylic acids, were tested for their potency to [^{3}H]naloxone binding sites in rat brain membranes. All of the new derivatives displayed highly attenuated binding affinity. The relatively most potent analogues were further investigated for their selectivity to μ- versus δ-receptors. The ligands we examined showed attenuated potency to both receptor types with a smaller decrease in the case of δ–binding. Trans position of the β-amino groups resulted in higher binding affinities then that of the corresponding cis isomers, the latter being more flexible than the former. It is concluded that the conformational constrains caused either by a rigid ring structure or cis isomers instead of D-Ala^2 in dermorphin derived peptides are unfavorable for binding activity to either opioid receptors. We propose that the interaction of the larger heptapeptide derivatives of dermorphins with the μ-receptor is distinct from that of the tetrapeptide morphiceptin.

Met^5-enkephalin-Arg^6-Phe^7 (Tyr-Gly-Gly-Phe-Met-Arg-Phe, MERF) is a naturally occurring heptapeptide that binds to opioid and non-opioid recognition sites in the central nervous system. Two synthetic analogues with single or double amino acid substitutions were prepared by solid phase peptide synthesis to achieve proteolytically more stable structures: Tyr-*D-Ala*-Gly-Phe-Met-Arg-Phe **(I)**, Tyr-*D-Ala*-Gly-Phe-*D-Nle*-Arg-Phe **(II)**. In this study, the potency of MERF and its two new derivatives were measured by ligand stimulated [^{35}S]GTPγS (guanosine-5'-O-(3-[γ-[^{35}S]thio)triphosphate) binding in rat brain membranes. The EC_{50} values of analog **(II)** were the highest in both tissues. Analog **(I)** was as effective as MERF in rat brain membranes, but showed a decreased potency in frog brain preparation. Again analog **(II)** seemed to be the least efficacious peptide, stimulating [^{35}S]GTPγS binding only by 59%. Specificity of the peptides was further

investigated by the inhibition of agonist-stimulated [^{35}S]GTPγS binding in the presence of selective antagonists for the opioid receptor types. The μ-selective antagonist cyprodime displayed the lowest potency in inhibiting the effects of the peptides, whereas norbinaltorphimine, a κ-selective, and naltrindole, a δ-selective antagonist were quite potent in both tissues. We concluded that MERF and its derivatives activate G-proteins mainly via κ- and δ-opioid receptors.

Prolonged exposure of cells to agonists induces adaptive changes resulting in reduced responsiveness to this ligand. The molecular mechanisms of opioid tolerance and dependence that result from chronic opioid agonist exposure are still not well understood.

We have studied if desensitization and/or internalization of μ-opioid receptors play a role in these phenomena by chronically treating rats *in vivo* by opioid agonists. Agonist-induced internalization of μ-opioid receptors was examined by radioligand binding with [^{3}H]DAMGO and subcellular fractionation of rat brain homogenates. Coupling of μ-opioid receptors to G-proteins was assessed by measuring the ability of DAMGO (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol) to stimulate [^{35}S]GTPγS incorporation into the α-subunits of G-proteins. Brain homogenates were subjected to subcellular fractionation to prepare highly purified synaptic plasmamembranes (SPM) and a light vesicular (LM) fraction, the latter representing intracellular binding sites. Etorphine at 0.4 mg/kg induced μ-opioid receptor desensitization and internalization. When analgesic tolerance was induced by morphine injection *subcutaneously* at increasing doses twice daily for 5 days the number of surface μ-opioid binding sites was not changed, however increased by 65% in LM. While DAMGO-stimulated [^{35}S]GTPγS binding was not significantly attenuated implying the lack of desensitization in SPMs, enhanced coupling of receptors to G-proteins was noted in the LM fraction after prolonged morphine exposure. Repeated *intracerebroventricular* injection of DAMCK (Tyr-D-Ala-Gly-(NMe)Phe-CH_2Cl) at 10 μg for 8 days twice daily decreased the maximal number of μ-opioid receptors by 20-28% in SPM with a concomitant increase of [^{3}H]DAMGO binding in LM. DAMGO produced similar but weaker changes than DAMCK. No desensitization was noted by measuring DAMGO-stimulated [^{35}S]GTPγS binding after DAMGO/DAMCK treatments. In conclusion, this part of the thesis demonstrates that the regulatory changes of the endogenously expressed μ-opioid receptors in rat brains display ligand-specificity following *in vivo* opioid exposure.

1. INTRODUCTION

1.1. The role of receptors in cell-to-cell communication

The cells of various tissues or organs have an elaborate cell-to-cell communication network that coordinates the growth, differentiation and metabolism of animals or human being. However, the communication is realized by a direct cell-to-cell contact in the case of only a few cell types, most of the cells communicate over long distances. The signaling cell releases a substance that is recognized by the target cell, in which it induces an appropriate response: hormone release, enzyme activation or gene transcription leading to protein synthesis. The signaling molecules are detected by specific **receptor** proteins. Receptors of the hydrophilic and large lipophilic molecules can be found on the cell surface and there are also binding sites in the cytosol or in the nucleus for small lipophilic ligands that can diffuse through the plasmamembrane.

Surface receptors mediate extracellular information to the cell and must generate an output. According to their output these receptors can be classified into four groups: 1) receptors which interact with guanine nucleotide binding proteins (G-proteins); 2) receptors with inherent tyrosine kinase activity; 3) receptors that form ion channels; 4) receptors with an unknown output.

The receptor function has two aspects: **binding specificity** of the receptor for the ligand and **effector specificity** for the resulting change in cellular behaviour. One of the most important binding properties of a molecule is the **binding affinity** which characterizes the ability of the ligands to bind to the receptor and offers the possibility to distinguish the binding properties of various molecules.

Ligands of a certain receptor can act either as **agonists** that bind to the receptor and cause the normal response or as **antagonists** that also bind to the receptor but do not activate an agonist-induced function. Agonists and antagonists display opposite effects, although they have closely related chemical structure. The different structure-activity relationships can be explained by the different functions served on interacting with the binding sites. The main differences between agonist and antagonist activity are summarized in **Table 1.1.** (Hruby et al. 1995).

Table 1.1. Differences between agonist and antagonist activity

Agonist	Antagonist
Binds to specific site in the receptor	Binds to the agonist site (competitive) or to some other site (non-competitive) on the receptor
Leads to a change in the receptor	Need not lead to change in receptor conformation, but if it does, it must be an inactive conformation
Often leads to phenomena such as patching, desensitization, etc.	Generally does not lead to patching, desensitization, etc.
Residence time on the receptor may be long or short	Generally requires long residence time on the receptor to be effective

(Hruby et al. 1995).

1.2. The opioid receptors

Perception of the pain is one of the most ancient functions of the nervous system. It plays a role in the adaptation to the environmental changes and can also indicate the serious irregular mechanisms, diseases developed in the organism. Plant extracts with analgesic effects (loss of pain sensation and diminished concern about pain) have been discovered by an empirical way for thousands of years. The major active ingredient of **opium** obtained from the poppy head was isolated by the German pharmacist Sertürner in 1805 and named **morphine** after Morpheus, the god of dream in the Greek mythology. Besides the analgesia several effects may be produced when morphine is administrated to human subjects who had not been treated previously or recently with the drug, including sedation, respiratory depression, decreased gastrointestinal motility and the sense of well-being (euphoria, the erasing of concerns and the diminishing the anxieties). These latter effects play a crucial role in the manifestation of psychical and physical dependence which can develop by chronic use of opiates for either medical or nonmedical reasons, and lead to dramatic changes of the behaviour and the function of the organism (see also in chapter 1.5.). 'Craving' for the sense of euphoria induces people to get and consume drugs despite its noxious and destructive effects. The other reason for the abuse of opiates is the desire to alleviate withdrawal reactions. The addiction to opiates became one of the most serious problems of the societies and represents an important subject of medical and biochemical research for many years.

1.2.1. Cloning of opioid receptors

The multiple effects of opioid ligands are mediated by **opioid receptors** that are specific cell-membrane receptors in the opiate sensitive neurons of the central nervous system. The existence of three major **opioid receptor types - μ, δ and κ-** is accepted (**Table 1.2.**).

Table 1.2. Heterogeneity of opioid receptors

Receptor	μ	δ	κ
Prototype ligand	**morphine**	enkephalins	Ethylketocycla-zocine (EKC)
Endogenous ligand	β-endorphin endomorphins **dermorphins**	[Met^5]enkephalin [Leu^5]enkephalin deltorphins	dynorphin A
Selective agonists	**DAMGO** morphiceptin sufentanyl PLO17	DADLE DPDPE DSLET D-Ala^{2}deltorphins	U50,488 U69,593 ICI197,067
Antagonist	naloxone	naloxone	naloxone
Selective antagonists	CTAP **cyprodime**	ICI174,864 **naltrindole** TIPP	**nor-binaltorphimine (nor-BNI)**
Effects	Analgesia, Euphoria, Respiratory depression, release of Prolactin	Analgesia Hypotension	Analgesia Dysphoria Diuresis Release of ADH
Experimental objects	Rat brain Rabbit cerebellum Guinea-pig ileum	Rat brain NG-108-15 cell line Mouse vas deferens	Rat brain Rabbit cerebrum Frog brain Guinea-pig cerebellum

Molecules that are used in our experiments are shown in bold type.

For each of the μ-, δ- and κ-opioid receptors only one gene has been cloned (MOR1, DOR1, KOR1) (Evans et al. 1992; Kieffer et al. 1992; Chen et al. 1993; Minami et al. 1993; Yasuda et al. 1993). Recently a new type of cDNA has been identified and has been named

ORL_1 (opioid receptor-like). This gene has a high degree of homology to the 'classical' opioid receptors (Mollereau et al. 1994). Numerous separately identified sequences encoding for the opioid receptors has been described to date. The tissue source of the cDNA libraries were from NG108-15 cell line (Evans et al. 1992; Kieffer et al. 1992), rat- (Chen et al. 1993; Abood et al. 1994), mouse- (Yasuda et al. 1993), guinea pig brain (Xie et al. 1992) and human samples (Wang et al. 1993; Knapp et al. 1994). Further multiplicity has not been confirmed yet by the cloning experiments (Raynor et al. 1994; Uhl et al. 1994).

1.2.2. *The structure of opioid receptors*

Opioid receptors are members of the membrane-bound neuroreceptor family that are functionally coupled to G-proteins. These receptors contain a single polypeptide chain and seven hydrophobic transmembrane domains. Cloned μ-, δ- and κ-opioid receptors consist of 398, 372 and 380 amino acids, respectively, in rat and mouse (for a review, see Benyhe et al. 1994; Kieffer et al. 1995). Much has been learned about the structural features of opioid receptors since the first successful cloning by Kieffer et al. (1992) and Evans et al. (1992) The amino acid sequences of μ- and δ-receptors are approximately 60-70% similar with the primary variations occurring in the extracellular N-terminus and the intracellular C-terminus domains and the external loops (Thompson et al. 1993; Reisine and Bell, 1993; Surratt et al. 1994). The 2^{nd} and 3^{rd} transmembrane domains of opioid receptors contain conserved aspartate residues (Surratt et al. 1994) that are believed to form an ion pair with the positively charged nitrogen atom which is a strict structural requirement common to all opioid ligands (for a review, see Morley, 1980). These conserved aspartic acid residues play a role in the morphine and enkephalin binding in the third transmembrane segment (for a review, see Benyhe et al. 1994). The second transmembrane region can determine the pharmacological specificity of the opioid receptors. The amino terminal region contains potential sites for asparagin-linked glycosylation. Ser and Thr are located in the third cytoplasmic loop and in the carboxy terminal region which can be substrate of different protein kinases. The second and third intracellular domain may bind to G-proteins.

In analogy with other G-protein coupled receptors, it was suggested that an opioid 'binding pocket' is formed by the seven putative α helices, while their extracellular loops would discriminate various classes of opioid ligands and define their μ-, δ– and κ–selectivity.

However, recent studies utilizing chimeric receptors and mutation studies combined with 3-D computer modeling have revealed the existence of multiple interactions throughout the receptor molecule which display a unique binding pattern for each ligand at least in the case of δ–receptor interaction (Kieffer et al. 1992; Befort et al. 1996).

1.2.3. The multiplicity of opioid receptors

The three classical types of the opioid receptors (μ, δ and κ) were further investigated by radioligand binding studies using rat brain membrane homogenates. On the basis of these experiments two subdivisions of μ-opioid receptors have been proposed (μ_1, μ_2) by Pasternak et al. (1986). It has been shown that the antagonist, naloxonazine discriminates between these two subtypes. To the different μ-receptors distinct physiological activities can be connected.

Two subdivisions of δ-opioid receptors (δ_1, δ_2) have been suggested (Jiang et al. 1991). Significant differences between the effect of several agonist and antagonist have been observed on the δ_1-, δ_2-receptors.

The subdivisional classification of the κ-opioid receptors seems to be the most complex. Non-homologous binding sites were suggested using [^{3}H]EKC in binding studies and κ_1- and κ_2-sites have been distinguished considering their different sensitivity to DADLE in guinea pig spinal cord (Attali et al. 1982). κ_2-sites are also characterized as a benzomorphan-preferring subset of κ-opioid receptors (Zukin et al. 1988; Wollemann et al. 1993). Recently the definition of the subtypes has been refined using the selective κ-agonist U-50,488. Considering these findings it has been reported that the high-affinity, U-69,593 sensitive κ_1-binding site predominates in guinea pig brain and the low affinity sites seemed to be insensitive to U-69,593 and predominated in rat brain (Zukin et al. 1988). Besides radioligand binding assays neurochemical and neuroendocrine studies of rat (Iyengar et al. 1986), electrophysiological experiments of mouse dorsal root ganglia cells (Fan et al. 1991), and antinociceptive measures of rodents (Gistrak et al. 1989) supported the existence of these two subtypes. Subsequently another study suggested the existence of the so called κ_3 sites, but this subtype is poorly defined in both molecular and pharmacological terms (Wollemann et al. 1993).

1.2.4. Opioid receptor distribution

Opioid receptors are widely distributed in brain. They are also found in spinal cord and peripheral sensory and autonomic nerves. In the supraspinal system **μ-opioid** receptors are localized in the neocortex, caudate-putamen, nucleus accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, nucleus tractus solitarius, spinal tegmental nucleus and dorsal horn. The distribution of the **δ-opioid** receptors is more restricted. The densest areas are olfactory-related neuronal areas, neocortex, caudate-putamen, nucleus accumbens and amygdala. **κ-opioid** receptors are distributed in an intermediate number in the caudate-putamen, nucleus accumbens, amygdala, hypothalamus, neuronal lobe of the pituitary, median eminence and nucleus tractus solitarius (for a review, see Mansour et al. 1988).

Opioid receptors that are present in the forebrain play a role in the behavioural reinforcement, in the expression of emotions and in anxiety. The endogenous opioids located in the hypothalamus are involved in the control of hormonal secretion, temperature regulation and fluid balance. Opioids also influence autonomic and gastrointestinal nervous system function as well as respiration and cardiovascular regulation (Mollereau et al. 1988; Konig et al. 1996; Walker and Koob, 1997).

Previous subcellular localization studies revealed that specific opioid binding sites can be detected in the synaptic plasmamembrane (SPM) and also in the light vesicular (LM) fraction in rat brain. The latter fraction also called 'microsomal' fraction, was enriched in endoplasmic reticulum, Golgi-membranes, and clathrin-coated vesicles (Roth et al. 1981; Bennett et al. 1985). The most prominent difference between the two pools was that μ-opioid receptors were coupled to G-proteins in the SPM, but displayed attenuated guanine nucleotide sensitivity in the LM (Szűcs and Coscia, 1992).

The relative proportion of opioid receptor types and subtypes in various species shows high differences. Frog brain represents a rich source of κ-opioid receptors, in guinea pig cerebellum κ_1-opioid receptors are presented and in rat brain μ-opioid receptors predominate (Schiller P.W. 1991; Benyhe et al. 1999).

1.3. The opioid ligands

1.3.1. Effects and selectivity of opioid ligands, peptide design

1.3.1.1. Endogenous ligands

The mammalian endogenous opioid ligands can be classified into four groups according to the precursors: proopiomelanocortin, proenkephalin, prodynorphin and pro-nociceptin/orphanin FQ (Nakanishi et al. 1979; Kakidani et al. 1982; Noda et al. 1982; Meunier et al. 1995) (**Table 1.3.**). Previous binding experiments performed with rat brain membranes and recent data with recombinant receptors suggest that no opioid peptide family can be exclusively associated with a specific opioid receptor type (Mansour et al. 1995). The exceptions are the very recently discovered endomorphin-1 and endomorphin-2 which are highly selective for μ-opioid receptors (Zadina et al. 1997). The amphibian skin consist of two families of D-amino acid-containing peptides; dermorphins and deltorphins. These are highly receptor selective peptides. The heptapeptide **dermorphin** displays remarkable affinity for μ-opioid receptors, deltorphins being δ-selective (Erspamer et al. 1989). The naturally occurring dermorphins contain an N-terminal tripeptide sequence Tyr-D-Xaa-Phe which is also present in deltorphins. A study by Charpentier et al. suggests that this common sequence motif which is topologically distinct from the region (C-terminal 'address' domain of the ligands) ensuring site-specific interactions with the receptors is recognized by both μ- and δ–opioid receptors (Charpentier et al. 1991). Tyr^1, Pro^2 and Phe^3 are the important residues for the biological activity of one of the most selective agonists for the μ-receptor, morphiceptin (Yamazaki et al. 1991; Mierke et al. 1990; Liebmann et al. 1986). By contrast, the endogenous mammalian opioid peptides (enkephalins, endorphins, dynorphins) are characterized by an identical N-terminal 'message' tetrapeptide sequence Tyr-Gly-Gly-Phe (Mansour et al. 1995; Portoghese, 1989). Keeping in mind that, besides the structurally diverse μ-receptor preferring peptide ligands, the prototypic μ-ligands are the morphine-like opiate alkaloids, one might assume that there are multiple factors determining the binding selectivity of μ-specific ligands.

Table 1.3. Endogenous opiod peptides

Precursor	**Endogenous peptide**	**ligand specificity**
Proopiomelanocortin (POMC)	β-Endorphin	$\mu > \delta >> \kappa$
Proenkephalin A	[Leu^5]enkephalin	$\delta > \mu >> \kappa$
	[Met^5]enkephalin	$\delta \sim \mu >> \kappa$
	[Met^5]enkephalin-Arg^6-Phe^7 (MERF)	$\kappa_2 > \delta > \mu > \kappa_1$
Prodynorphin (Proenkephalin B)	Dynorphin A (1-17)	$\kappa >> \mu > \delta$
	Dynorphin A(1-13)	$\kappa > \mu \sim \delta$
	Dynorphin A (1-8)	$\kappa > \mu \sim \delta$
	α-neoendorphin	
	β-neoendorphin	
Pronociceptin/ Orphanin FQ	Nociceptin	ORL_1
Proendomorphin *	Endomorphin-1	μ-selective
	Endomorphin-2	μ-selective
	Dermorphin	μ-selective
	Dermenkephalin	δ-selective
	[D-Ala^2]-deltorphin I	δ-selective
	[D-Ala^2]-deltorphin II	δ-selective

* Presumed to exist, awaiting discovery (Corbett, 1993; Borsodi and Tóth 1995)

From the point of view of this study it is important to emphasize the posttranslational products of pro-enkephalin. Met- and Leu-enkephalin are processed from pro-enkephalin and display high affinity for δ-opioid receptors, ten-fold lower affinities for μ-opioid receptors and have a decreased preference for the κ-opioid receptors (Corbett, 1993).

Interestingly, the Met-enkephalin derived endogenous opioid peptide **Met^5-enkephalin-Arg^6-Phe^7** (Tyr-Gly-Gly-Phe-Met-Arg-Phe; MERF) have shown notable affinity towards κ_2-opioid receptors (Wollemann et al. 1994; Benyhe et al. 1999). MERF was

discovered in chromaffin granules of bovine adrenal glands and also in beef striatal extracts (Stern et al. 1979). The peptide is cleaved from proenkephalin-A and localized to the mammalian central nervous system (Kilpatrick et al. 1983). MERF has also been found in relatively high concentrations in the brain of lower vertebrates (Rossier et al. 1980). The heptapeptide has been reported to be antinociceptive (Inturrisi et al. 1980), and more recently its antitussive effect has been noted (Kamei et al. 1994).

Opioid receptor binding properties of MERF have been studied in various species using labelled oripavine- (^{3}[H]etorphine) and benzomorphan-type (^{3}[H]ethylketocyclazocine) ligands (Attali et al. 1982; Attali et al. 1982; Benyhe et al. 1990). These studies have led to the conclusion that MERF binds mainly to the so-called κ_2-binding sites (Zukin et al. 1988; Wollemann et al. 1993). In a more direct approach, MERF has been radiolabelled by catalytic dehalotritiation resulting in [^{3}H]MERF with high specific radioactivity (Wollemann et al. 1994). Using this compound as a radioprobe it has been demonstrated that [^{3}H]MERF is able to interact selectively with the κ_2-receptors in frog (*Rana esculenta*) brain membranes (Wollemann et al. 1994). In rat brain membranes κ- and δ-opioid receptors, and non-opioid binding sites have also been shown to be specifically labelled by [^{3}H]MERF (Benyhe et al. 1997). The radiopeptide binds only to the non-opioid sites in guinea pig cerebellar membranes, where no κ_2- but κ_1-opioid receptors are present (Zukin et al. 1988).

1.3.1.2. Synthetic opioid ligands

In order to examine the unique physiological role of individual receptor types, linear analogues of enkephalins, β-casomorphins, dermorphins with high site specificity have been developed. Two important synthetic μ-selective peptides are **DAMGO** (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol) end its chloromethyl-keton derivative **DAMCK** (Tyr-D-Ala-Gly-(NMe)Phe-CH_2Cl). The latter binds irreversible to the μ-binding sites and has been used for affinity labeling to determine the molecular weight of the μ-opioid receptor (Benyhe et al. 1987; Varga et al. 1988; Oktem et al. 1991).

Mapping the specific structural requirements of individual receptor types and elucidation of their unique physiological role would require highly site-specific ligands. The pioneering work of Hruby at al. and Schiller et al. revealed that conformational constrains are

absolute requirements for achieving μ-selective peptide binding (Hruby V.J. and Hadley M.E. 1986; Schiller et al. 1991).

The design, synthesis and use of conformationally constrained analogues of peptides that is required for achieving more selective binding has grown enormously in recent years (Smith et al. 1991). Local constrains (Terenius L. 1997; Yamazaki et al. 1991) can be introduced in the peptide backbone by employing N^{α}-methylated amino acids (which will restrict the Φ torsion angle, Φ=-120° ± 20°), proline (Φ=-70° ± 20°,) or cyclic β-amino acids. Formation of mono- or polycyclic structures like disulphide bridges, cyclising the side-chain to the backbone or cyclising the C-terminus to the N-terminus also reduce the conformational freedom of peptides.

1.4. The signal transduction of opioid receptors

1.4.1. The role of G-proteins

The transmission of extracellular information that are carried by opioid ligands are mediated by opioid receptors interacting with heterotrimeric **guanine nucleotide binding proteins (G-proteins)**. These heterotrimeric G-proteins are members of a guanine nucleotide-binding protein superfamily as are cytoskeletal proteins and low molecular weight GTP-binding proteins (ras p21 protooncogenes and ras related proteins) (Spiegel, 1987; Gilman, 1987). The features of regulatory G-proteins include: 1) association with the cytoplasmic surface of the plasma membrane; 2) function as receptor-effector couplers; 3) heterotrimeric structure with subunits designated α, β and γ. These subunits are distinct gene products. To date 23 α-subunits (encoded by 17 different genes), 5 β-subunits and 12 γ-subunits have been cloned at this time (for a review, see Simon, 1991; Kehlenbach et al. 1994; for a review, see Nurnberg et al. 1995). The γ-subunits, in contrast to the β-subunits, show a high degree of diversity (Ray et al. 1995; Morishita et al. 1995). The α-subunits are subdivided into 4 main families: G_s, G_i, G_q, G_{12} (for a review, see M. Szűcs 1995).

α-subunits are required for the specificity in receptor-effector coupling, bind guanine nucleotides with high affinity and specificity, possess intrinsic GTP-ase activity, serve as substrates for ADP-ribosylation by bacterial toxins and directly regulate effector activity. **βγ-subunits**, coupled tightly but noncovalently to the complex, are responsible for receptor G-

protein coupling, directly modulate effector activity and can inhibit G-protein activation by blocking α-subunit dissociation (Spiegel A.M. 1990).

The **G_s-protein**, the stimulatory G-protein of adenylyl cyclase was discovered in 1977 by Pfeuffer and is responsible for mediating adenylyl cyclase activation.

The effect of opioid ligands are mediated by pertussis toxin sensitive **G_i and G_o proteins**. G_i-proteins were initially purified from rabbit liver (Bokoch et al. 1983) and human erythrocytes and can regulate a great variety of effector systems such as adenylate cyclase, phospholipase C, phospholipase A_2, phospholipase D, K^+ channels, Ca^{2+} channels. G_o proteins activate also ion channels (Spiegel A.M. 1990).

Agonist induced G-protein activation is demonstrated in **Fig. 1.1.**. When an agonist interacts with its receptor, the receptor associates with a specific heterotrimeric G-protein that is in the inactive, GDP bound form. The activated receptor induces the release of GDP from the α-subunit. The nucleotide free α-subunit binds GTP and induces conformational changes leading to 1) the dissociation of the G-protein from the receptor and 2) dissociation of the heterotrimer into GTP-liganded α-subunit and a βγ dimer. Both of them can regulate effector molecules including adenylyl cyclase and phospholipases as well as certain ion channels. Due to its GTP-ase activity the α-subunit restores the inactive form of the Gα-subunit and the termination of G-protein activation is achieved by the reassociation of the α-subunit with the βγ dimer. (for a review, see Birnbaumer et al. 1990; Birnbaumer, 1992). If non-hydrolysable analogues of GTP, e.g. [^{35}S]GTPγS are present in the reaction mixture the cycle can not terminate and the activation of the G-proteins becomes measurable. Taking into consideration the high number of G-protein coupled receptors, the diversity of the G-protein subunits and the fact that many receptors are capable of activating different subtypes of G-proteins, it has become evident that the signal transduction by heterotrimeric G-proteins provides a complicated but well specialized function. However, different type of receptors which mediate functionally similar responses often activate the same subtype of G-proteins. It is suggested that by the receptor-G-protein interaction signalling can also converge (for review, see Offermanns and Schultz, 1994).

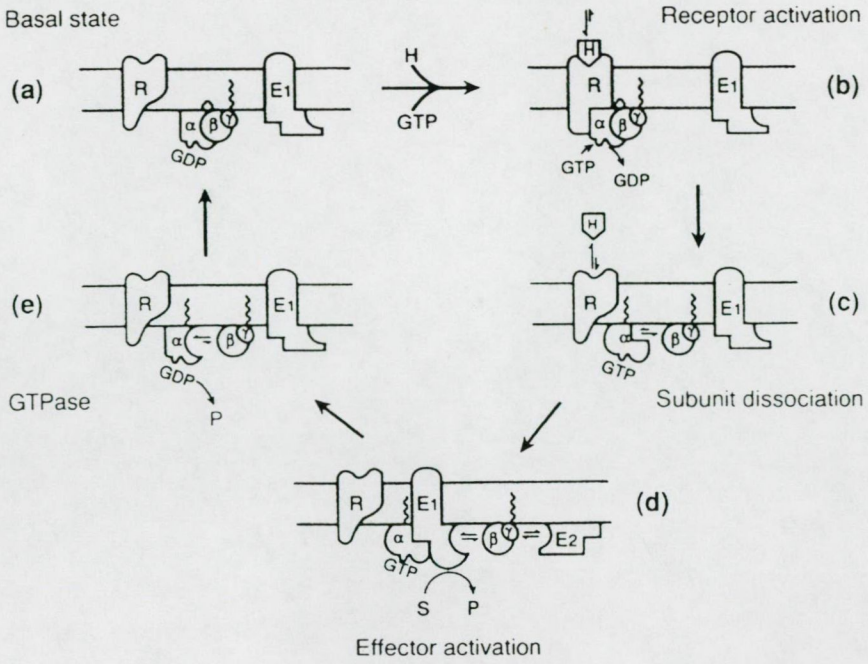

Fig. 1.1. GTPase cycle

1.4.2. Theoretical basis of agonist stimulated [^{35}S]GTPγS binding studies

In order to investigate the G-protein activation by agonist-liganded receptors in a quantitative manner, the binding of a radiolabeled GTP analog (guanosine-5'-O-(3-[γ-[^{35}S]thio)triphosphate, **[^{35}S]GTPγS)** to G-protein is determined. Agonist stimulated [^{35}S]GTPγS binding has been widely used for many G-protein coupled receptors and offers the possibility to determine the potency and efficacy of ligands for a given receptor and also to distinguish agonists from antagonists. [^{35}S]GTPγS is available with relatively high specific radioactivity (1000-14000 Ci/mmol; physical half-life 87.4 days) (Weiland and Jakobs, 1994).

Agonists differ in their maximal ability to cause biological responses. The term **efficacy** has been introduced first by Stephenson (1997). In the case of G-protein coupled receptors it is determined by two signal transduction components: 1) the ability to induce receptor stimulated guanine nucleotide exchange on the G-protein α-subunit 2) the number of G-proteins activated by occupied receptors (Selley et al. 1997). **Intrinsic efficacy** is characteristic of a certain drug and in contrast to the efficacy, is independent of receptor density. It represents the amount or response induced per unit of receptor occupation (Johnson and Fleming, 1989). **Full agonists** are able to produce maximal response without occupying all the available receptors. In the case of the classical **partial agonists** the

activation of the whole receptor pool is required to produce the maximal effect. Drugs with modest intrinsic efficacy can be partial agonists in a tissue with relatively low receptor concentration and full agonists in another tissue with higher density of the same receptors. This type of ligands are called **mixed full/partial agonists** (Johnson and Fleming, 1989; Selley et al. 1997).

Agonist induced stimulation of G-protein activation has only been observed in the presence of **GDP** which seems to act by enhancing the ratio between agonist-stimulated and basal level of activity (Breivogel et al. 1998).

Initially the G-proteins are in a GDP-liganded form and the agonist independent binding of [^{35}S]GTPγS to G-proteins is limited by the dissociation of GDP from the binding sites. Addition of GDP keeps the G-proteins in the GDP-liganded form and decreases basal [^{35}S]GTPγS binding more then agonist stimulated binding. The latter has been caused by the fact that in the presence of agonist G-proteins have lower affinity for GDP than in the absence of agonist and due to these affinity differences GDP competes with [^{35}S]GTPγS significantly better at unactivated G-proteins than agonist-activated G-proteins.

Opioid agonists display differential abilities to stimulate [^{35}S]GTPγS binding maximally in the presence of GDP. The efficacy differences can be magnified by increasing GDP concentrations. The potency of GDP in inhibiting agonist stimulated [^{35}S]GTPγS binding seemed to be inversely proportional to the efficacy of the agonist. GDP stabilizes G-proteins in the inactive state. The agonists of higher efficacy may be less affected by this stabilizing effect of GDP in their ability to activate G-proteins because these agonists produce a higher GTP-binding state in the guanine nucleotide binding sites of the G-protein α-subunits, than agonist of lower efficacy (Weiland and Jakobs, 1994; Selley et al. 1997; Breivogel et al. 1998).

NaCl has opposite effect on G-protein coupled agonist-free and agonist occupied receptors. The addition of sodium ions into the [^{35}S]GTPγS binding assay leads to the uncoupling of unoccupied receptors, but not the occupied receptors, from their respective G-protein. This process results in a reduction of the basal G-protein activity and enhances the signal to noise ratio. Increasing concentration of sodium ions decrease the maximal stimulation of partial agonists. At low sodium ion concentration partial agonists can behave as full agonists. The additional presence of sodium ions increases the EC_{50} values of full agonist but has no effect on the maximal stimulation (Williams et al. 1997).

1.4.3. Effector mechanisms

It seems that most type of cloned opioid receptors belong to the G_i/G_o -coupled superfamily of receptors and share several common properties in their ability to interact with second messenger systems. The direct G-protein ($\beta\gamma$ or α subunit) mediated effects are: 1) inhibition of adenylyl cyclase, 2) activation of an inwardly rectifying potassium channel; 3) inhibition of voltage operated calcium channels; 4) regulation of phospholipase C (PLC) activation (for a review, see Simonds, 1988; Childers, 1991; Gilman, 1987).

1) One of the best studied signalling pathways initiated by opioid ligands is the opioid receptor-inhibited adenylyl cyclase. At least 8 types of adenylyl cyclase has been characterized. Some (or all) of them are inhibited by receptors that activate G_i and G_o proteins. The G_i mediated inhibition of adenylate cyclase might occur on two distinct ways: 1) $\beta\gamma$-subunits, released from activated G_i, inhibit the dissociation of $G_{\alpha s}$; 2) GTP-bound $G_{\alpha i}$ directly inhibit the catalytic unit of adenylate cyclase (Levitzki A. 1990; Milligan G. 1990; Spiegel A.M. 1990; Birnbaumer, 1992).

2) Increase in inwardly rectifying potassium conductance caused by the stimulation of μ-opioid receptors results in a membrane hyperpolarization and decrease the neuronal firing rate in locus coeruleus and hippocampal neurons (North et al. 1987; Miyake et al. 1989; Wimpey and Chavkin, 1991; Williams et al. 1988). This effect was inhibited by the opioid antagonist naloxone implying that the μ-opioid receptors are functionally coupled to potassium channel. cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), the most widely studied kinases regulate differently the μ-opioid receptor coupling to the G-protein-activated potassium channel (Chen and Yu, 1994).

3) Coupling of opioid receptors to a variety of voltage dependent Ca^{2+} channel (L and N type) mediated probably by the $\beta\gamma$-subunit liberated from G_i/G_o-proteins (Piros et al. 1996) and also by $G_o\alpha$ (Milligan G. 1990).

The combined activation of potassium channels and the inhibition of Ca^{2+} channels by opioid ligands results in a reduced neurotransmitter release from presynaptic terminals (North et al. 1983).

4) It has been reported that the tree types of opioid receptors activate PLC via $\beta\gamma$-subunit of the G_i/G_o-proteins and stimulate the cleavage of phosphoinositol-4,5-diphosphate (PIP_2) to inositol-1,4,5-triphosphete (IP_3) (Smart et al. 1995). It is also demonstrated that μ-opioid receptor agonists activate PLC via Ca^{2+} channel opening in SH-SY5Y human neuroblastoma cells (Smart et al. 1995).

1.5. Opioid tolerance and dependence

1.5.1. Tolerance, dependence, withdrawal

Tolerance develops after chronic (sustained or repeated) opioid drug treatment and refers to the decrease of the sensitivity of the biological system to an opiate (for a review, see Cox B.M. 1993; for a review, see Trujillo and Akil, 1991). Prolonged administration of opioid drugs also leads to **psychical and physical dependence**, a persistent relapsing disorder. The former reflects the special behaviour and 'craving', an incentive to satisfy the appetite for drug (Terenius L. 1997). The latter refers to the altered function of the organisms influenced by the drug whereby further administration is necessary to avoid physical disturbance. Termination of drug-treatment leads to **'withdrawal'** syndromes. This hyperexcitable state shows typically opposite signs to the acute actions of the drug, including restlessness, irritability, tremor, diarrhoea, goose flesh and muscle cramps (Trujillo and Akil, 1991).

The molecular mechanisms underlying opioid tolerance/dependence are not fully understood despite intense research (for a review, see Nestler et al. 1993). It is generally hypothesized that desensitization and/or internalization of the receptors, among others, should play a role in opioid tolerance.

1.5.2. Desensitization, internalization and down-regulation

Prolonged exposure of cells to agonists results in a loss of responsiveness to further agonist stimulation through a process termed **desensitization**. The β-adrenerg receptors undergo phosphorylation during the desensitization process and it leads to functional uncoupling of the receptor from the effector system in turkey erythrocytes (Sibley et al. 1984). Receptor-G-protein uncoupling requires minutes to occur from the exposure to ligands.

Internalization or sequestration is generally envisioned as movement of the receptor into a cell compartment distinct from the plasma membrane (reviewed in Harden, 1983; for a review, see Mahan et al. 1987). This membrane compartment is essentially defined by two criteria; it is not accessible to hydrophilic ligands, and it has a lower density than plasma membranes, allowing separation of the two compartments by sucrose density gradient centrifugation (Stadel et al. 1983; Strader et al. 1984). Internalized receptors can be either stored or destroyed in intracellular membrane compartments (coated vesicles, endosomes and lysosomes, resp.) or recycled to the cell surface.

After chronic agonist exposure receptor **down-regulation** might also occurs, which entails a decrease in the total pool (surface + intracellular) of receptors. This process develops more gradually and becomes significant after hours of agonist treatment (Strader et al. 1984; Sibley and Lefkowitz, 1985; Koenig and Edwardson, 1997).

Down-regulation usually entails receptor internalization by **endocytosis** and subsequent degradation.

It has been reported that opioid tolerance involves uncoupling of the receptors from the G-proteins (Law et al. 1983). Previous studies revealed that opioids are able to change the subcellular distribution of μ-opioid receptors in cultured cells and also in the peripheral and central nervous system (Arden et al. 1995; Keith et al. 1998; Sternini et al. 1996). The rapid process of receptor internalization has been observed for μ-opioid receptors in transfected cells and recently in neurons *in vivo* (Arden et al. 1995; Sternini et al. 1996; Keith et al. 1998). It has been shown, however, that whereas enkephalins and the alkaloids, morphine and etorphine, activated the receptor via the same signalling pathway, only **enkephalins** and **etorphine** induced rapid endocytosis, **morphine** failed to do so (Burford et al. 1998). Enkephalins and etorphine, but not morphine, provoked the translocation of μ-opioid receptors into early endosomes (von Zastrow and Kobilka, 1994; Keith et al. 1996) suggesting that these receptors are internalized via an endocytic pathway similar to that of other G-protein coupled receptors (GPCR) (von Zastrow and Kobilka, 1992).

Both **DAMGO** and **DAMCK** produced time- and concentration-dependent, naloxone-reversible antinociception and also tolerance developed after chronic treatment (Szabó et al. 1999). Previous studies have shown that DAMGO caused internalization of the μ-opioid receptors in HEK cells (Burford et al. 1998). Down- regulation has been so far demonstrated in primary cultures of tumour tissue, fetal brain and host cells expressing recombinant receptors (for reviews see Cox B.M. 1993; Zadina et al. 1995). Chronic DAMGO exposure induced desensitization and down-regulation of the μ-opioid binding sites in C6 glial cells (Yabaluri and Medzihradsky, 1997).

Ko et al. (1999) has shown down-regulation of δ-opioid receptors in the neuronally derived Neuro2a cells. Recently, agonist-specific differences in the regulation of the rapid endocytosis of κ-opioid receptors have also been noted (Li et al. 1999).

The opioid ligand efficacies in agonist-induced μ-receptor phosphorylation and desensitization generally paralleled their efficacies in opening ion channels and inhibiting adenylyl cyclase (Yu et al. 1997).

2. THE AIM OF THE STUDIES

The complex effect of the opioid ligands are mediated by opioid receptors which are functionally coupled to heterotrimeric G-proteins. The first level of testing opioid ligands is to determine their affinities and selectivity for the opioid receptor types. The second level of the characterization is to examine the ability of the ligands to activate G-proteins. The third level is to investigate the receptor regulation induced by agonist treatment. In this work these three main features of opioid ligands have been studied using radioligand binding experiments and ligand stimulated [^{35}S]GTPγS binding assays.

I. In the first part eight conformationally constrained analogues of the μ-specific opioid peptide dermorphin were tested for their potency to bind to opioid binding sites in rat brain membranes.

A) The receptor binding affinity of the new ligands was tested in competition experiments.

B) The relatively most potent derivatives were analysed for their selectivity to μ- and δ-opioid receptors.

II. In the second part the potency of Met^5-enkephalin-Arg^6-Phe^7 and its derivatives (**I:** Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe, **II:** Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe) were investigated in frog and rat brain membranes.

A) The ability of the peptides to induce receptor-G-protein interactions was examined in the two systems.

B) The receptor type specificity of Met^5-enkephalin-Arg^6-Phe^7 and its analogues was analysed using opioid antagonists.

III. In the third part animals were treated *in vivo* with opioid agonists (etorphine, morphine, DAMGO, DAMCK) that differed in their chemical structure, abuse potential and efficacy. The regulatory changes induced by various μ-opioid agonist treatments were examined in rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions. It was of interest to see whether the regulation of μ-opioid receptors *in vivo* displays ligand specificity.

A) Changes in the intracellular distribution of μ-opioid receptors were determined.

B) The degree of desensitization was investigated by measuring the receptor-G-protein functional coupling.

3. MATERIALS AND METHODS

3.1. Chemicals

Radiochemicals [^{3}H]Ile^{5,6}-deltorphin II (49.5 Ci/mmol) was synthesized in the Isotope Laboratory of the BRC (Nevin et al. 1994). Radiolabeled guanosine-5'-O-(3-[γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (37-42 Tbq/mmol) was purchased from Isotope Institute Ltd., (Budapest, Hungary); [^{3}H]naloxone (71.3 Ci/mmol) was prepared as described (Tóth et al. 1982); [^{3}H]MERF ([^{3}H]Tyr-Gly-Gly-Phe-Met-Arg-Phe, 40 Ci/mmol) was also synthesized in the BRC Isotope Laboratory (Szeged, Hungary) as described previously (Wollemann et al. 1994). [^{3}H]DAMGO were obtained from Multiple Peptide System (San Diego, CA, USA) via the Drug Supply Program of NIDA (Rockville, USA).

Opioid ligands Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) was obtained from Multiple Peptide System (San Diego, CA, USA) via the Drug Supply Program of NIDA (Rockville, USA). Tyr-D-Ala-Gly-(NMe)Phe-CH_2Cl (DAMCK) was synthesized by A. Magyar (ELTE, Budapest) as published (Benyhe et al. 1987; Varga et al. 1988). Naloxone hydrochloride was kindly donated by S. Hosztafi (Alkaloida Ltd., Tiszavasvári, Hungary). Cyprodime was synthesized by H. Schmidhammer (Institute of Organic and Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria) (Schmidhammer et al. 1989).

Dermorphin derivatives:

(I) cis-2-amino-cyclohexane carboxylic acid; (II) trans-2-amino cyclohexane carboxylic acid; (III) cis-2-amino-cyclohexene carboxylic acid; (IV) trans-2-amino-cyclohexene carboxylic acid; (V) cis-2-amino-norbornane carboxylic acid; (VI) trans-2-amino-norbornane carboxylic acid; (VII) cis-2-amino-norbornene carboxylic acid (VII) trans-2-amino-norbornene carboxylic acid were synthesized by G. Tóth and F. Fülöp (SZAOTE, Szeged, Hungary) as published (Bozó et al. 1997).

Synthetic MERF analogues:

I: Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe; **II:** Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe,

were prepared by solid phase peptide synthesis and purified by reversed phase high-pressure liquid chromatography by J. Farkas in the Isotope Laboratory of the BRC.

Other chemicals Norbinaltorphimine (nor-BNI) and naltrindole were from ICN-Alkaloida (Tiszavasvári Hungary); guanosine 5'-diphosphate (GDP), unlabelled guanosine-

5'-O-(3-thio)triphosphate (GTPγS), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), and NaCl were products of Sigma Chemicals, St. Louis, MO, USA. Sucrose was from Merck, Darmstadt, Germany.

3.2. Methods

3.2.1. Rat brain membrane preparation

A crude membrane fraction of rat brains was prepared according to Szűcs and Coscia (1990). Briefly, the animals were decapitated, their brains without cerebella rapidly removed and homogenized in 30 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer by a teflon-glass homogeniser. After centrifugation at 20,000 × g for 25 min at 4°C the resulting pellets were suspended in 30 volumes of the same buffer and incubated for 30 min at 37°C to remove endogenous opioids. Centrifugation was then repeated as described above. The final pellets were suspended in 5 volumes of 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at -70°C. Membranes were thawed before use, diluted with buffer and centrifuged at 20,000 × g for 25 min at 4°C to remove sucrose. The resulting pellets were taken up in appropriate fresh buffer and immediately used in radioligand- or [^{35}S]GTPγS binding experiments (see there). Protein content of the membranes was determined by the use of dye-protein binding reaction (Bradford, 1976).

3.2.2. Frog brain membrane preparation

Frog brain membranes were prepared as previously described (Simon et al. 1984). Briefly, whole brains were homogenized in 10 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA, 0.1 mM PMSF, 10 mg/ml bacitracin, and 40 kIU aprotinin (buffer A). The homogenate was centrifuged at 25,000 × g for 20 min at 4°C. The resulting pellet was suspended in the same buffer containing 0.32 M sucrose and stored at -70°C. Before use, the membrane fraction was thawn, diluted with buffer A and centrifuged at 25,000 × g for 20 min at 4°C to remove sucrose. Final pellets were resuspended in buffer A and used immediately for ligand binding assays and for G-protein stimulation.

3.2.3. Subcellular fractionation of rat brains

Subcellular fractionation of rat brains were according to Roth et al. (1981) and Szűcs et al. (1992). Briefly, fresh forebrains were gently homogenized and after repeated centrifugation of the homogenates at 1,000 x g the combined supernatants were centrifuged at 12,000 x g for 20 min The pellets were suspended in 10% sucrose, and consecutive centrifugations at 20,000 x g for 25 min and 14,000 x g for 20 min twice resulted in crude SPM. Crude microsomes were obtained from the 12,000 x g supernatant by consecutive centrifugations at 20,000 x g for 25 min and 128,000 x g for 1 h. Purified SPM fractions were resolved on a 10%, 28.5% and 34% sucrose density step gradient that was spun at 100,000 x g for 2 hrs, whereas 'light membranes' (LM) were obtained from a 10% and 28.5% gradient centrifuged at the same speed. Fractions collected from the gradient were diluted three-fold with TRIS-HCl pH 7.4, pelleted at 100,000 x g for 1 h, suspended in 50 mM TRIS-HCl pH 7.4 and immediately used for receptor binding or [^{35}S]GTPγS functional assay. Detailed characterization of the subcellular fractions by marker enzymes, electron microscopy and [^{3}H]DAMGO binding was reported earlier (Roth et al. 1981; Szűcs et al. 1992).

In the case of etorphine treatment, fractions collected from the sucrose gradients were supplemented with 5 μM GTP, 100 mM NaCl followed by incubation at 37°C for 30 min, dilution 3-fold with 50 mM TRIS-HCl pH 7.4 buffer and centrifugation at 128,000 x g for 1 h. Resulting pellets were suspended in Tris buffer, incubated at 37°C for 15 min and sedimented at 128,000 x g for 1 h. Final pellets were homogenized in 50 mM TRIS-HCl pH 7.4 buffer to yield about 0.3 mg protein/ml homogenate.

3.2.4. Radioligand binding studies

800 μl membrane suspension (≈0.2-0.4 mg protein) was incubated with the appropriate [^{3}H]labelled compounds in 1 nM concentration ([^{3}H]naloxone for 60 min at 0°C, [^{3}H]DAMGO for 60 min at 25°C, [^{3}H]Ile^{5,6}-deltorphin II for 45 min at 35°C) in the absence (total binding) or presence of 10^{-10}-10^{-4} M of the peptides to be investigated in a final volume of 1 ml. The nonspecific binding was determined with 10^{-5} M naloxone and subtracted from the total value to yield specific binding. The reactions were stopped by filtration through Whatman GF/B ([^{3}H]naloxone) or GF/C filters ([^{3}H]DAMGO and [^{3}H]Ile^{5,6}-deltorphin II) utilizing a Brandel M24-R Cell Harvester (Gaithersburg, MD, USA).

Filters were washed twice with 10 ml ice-cold 50mM Tris-HCl (pH 7.4) buffer and dried. Radioactivity was determined in a toluene-based scintillation cocktail in a Beckman LS 5000TD counter.

3.2.5. [^{35}S]GTPγS binding assay

Stimulation of [^{35}S]GTPγS binding. Unless otherwise indicated, frog or rat brain membrane fractions (≈ 10 μg of protein) were incubated in Tris-EGTA (50 mM Tris-HCl pH 7.4, 1 mM EGTA, 3 mM $MgCl_2$) buffer containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9}-10^{-5} M) of MERF, Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I)** and Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe or increasing concentrations (10^{-8} -10^{-4} M) of DAMGO in the presence of 100 μM GDP in a total volume of 1 ml for 60 min at 30°C according to Sim et al. (1995) and Traynor et al. (1995) with slight modifications (Fábián et al. 1998). Non-specific binding was determined with 10 μM GTPγS and subtracted. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/F filters with a Millipore manifold. Filters were washed with 3 x 5 ml ice-cold buffer, and radioactivity of the dried filters was detected in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Turku, Finland).

Inhibition of ligand stimulated [^{35}S]GTPγS binding. Frog or rat brain membrane fractions (≈ 10 μg of protein) were incubated in Tris-EGTA (50 mM Tris-HCl pH 7.4, 1 mM EGTA, 3 mM $MgCl_2$) buffer containing [^{35}S]GTPγS (0.05 nM) and 100 μM GDP in the absence (basal activity), or in the presence of 1 μM MERF in frog and 1 μM Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe in rat with increasing concentrations of the appropriate antagonists. Incubation and filtration were performed as described above.

3.2.6. *Treatment of animals*

***In vivo* drug treatments.** Wistar rats (250-280 g) were used. They were kept under a standard light-dark cycle (lights on between 0600 and 1800 h) with food and water available *ad libitum.* The animals were kept and treated according to the rules of the Ethical Committee for the Protection of Animals in Research (BRC and SZAOTE Szeged, Hungary).

Rats were injected *intraperitoneally (ip)* with 0.4 mg/kg etorphine or saline according to Sternini et al. (1996). Animals were killed 30 min later.

Morphine-HCl was administered *subcutaneously (sc)* twice daily in increasing doses for 5 days in a total dose of 330 mg/kg. Dry treatment and pharmacological assays were performed by M. Szikszay and G. Horváth (SZAOTE, Szeged, Hungary).

DAMGO and DAMCK were given *intracerebroventricularly (icv)*. Surgery, dry treatment and antinociception measurement with the tail-flick assay were performed by G. Szabó (SZAOTE, Szeged, Hungary) as published (Szabó et al. 1999). All *icv* treatments were delivered slowly to freely moving animals to prevent unspecific effects. The peptides were dissolved in artificial cerebrospinal fluid (CSF) and injected in a volume of 2 µl. Chronic administration of the drugs was started 5 days after *icv* cannulation either at 100 ng/kg once daily for 5 days for 'LOW-DOSE' treatments, or at 10 µg/kg twice daily for 8 days for 'HIGH-DOSE' treatments. Control animals were treated with CSF in parallel. To test the 'ACUTE' effect of the peptides, 100 ng/kg DAMCK or DAMGO was given, and the rats were sacrificed 20 min or 16 hrs later.

Animals chronically treated with morphine or DAMGO/DAMCK were killed 16 hrs after drug treatments. After each treatment, forebrains minus cerebella were quickly removed, put on ice followed immediately by subcellular fractionation.

3.4. Data analysis

Radioligand binding assays. The analysis of binding experiments is based on the so-called 'principle of mass action'. A ligand (L) interacts with a receptor (R) and forms a complex (LR) in a bimolecular reversible reaction.

$$R + L \leftrightarrow RL$$

$$[L][R]k_{+1}=[LR]k_{-1}$$

where k_{+1} is the association rate constant in units of M^{-1} min^{-1}

k_{-1} is the dissociation rate constant in units of min^{-1}

$$\frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_{+1}} = K_d$$

where K_d is the equilibrium dissociation constant

IC_{50} (Inhibitory concentration 50%) value is the half maximal inhibitory concentration of unlabelled ligand. K_i (inhibition constant, equilibrium dissociation constant for the competitive ligand) is defined as the concentration of the unlabeled ligand that binds to half the binding sites at equilibrium in the absence of radioligand. The relationship between IC_{50} and K_i values is described by the equation of Cheng and Prusoff (1973).

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

The competitive binding studies measure the binding of a constant dose of labelled ligand in the presence of various concentrations of unlabeled ligand. Binding data from homologous and heterologous displacement curves were analysed by means of the non-linear least-squares regression computer program 'LIGAND' to obtain K_i (inhibitory constant), K_d (dissociation constant) and B_{MAX} (number of binding sites) values (Munson and Rodbard, 1980).

Data are reported as mean ± S.E.M. of 3 or more experiments each performed in duplicate. Significant differences among values were determined using Student's t test. Due to relatively high inter-experiment variance in the B_{MAX} values and the small changes induced by dry treatments, more significant changes were obtained if significance was determined by comparing % changes in B_{MAX} from matched samples of treated vs control samples.

[^{35}S]GTPγS binding assay. EC_{50}, IC_{50} and maximal stimulation values were obtained from nonlinear regression analysis using the program Graph Pad Prism 2.01 and considering a sigmoidal dose response curve. Maximal stimulation is given in percent stimulation of [^{35}S]GTPγS binding over basal. EC_{50} values were defined as the concentration of DAMGO producing 50% of the maximal response. IC_{50} values were defined as the half-maximal inhibitory concentration of the antagonists. Data are reported as mean ± S.E.M. of 3-6 experiments each performed in triplicate.

4. RESULTS

4.1. Opioid binding activity of dermorphin analogues containing cyclic β-amino acids

4.1.1. Competition of dermorphin analogues for rat brain opioid receptors

In contrast to all mammalian opioid peptides, **dermorphin** (DMF, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), that was originally isolated from amphibian skin, contains a D-amino acid in the 2nd position of the sequence and a C-terminal carboxamide group and, therefore, it is relatively stable against enzymatic degradation (Erspamer et al. 1980). The natural heptapeptide shows remarkably high μ-selectivity which is comparable to that of β-casomorphins isolated from bovine milk (Brantl et al. 1981).

In the present work, 8 conformationally constrained analogues of dermorphins were synthesized by replacing D-Ala^2 with β-amino-cyclic carboxylic acids. The resulting peptides (**Table 4.1.**) were tested for their binding activity to μ- and δ–opioid receptors in rat brain membranes. In **Fig. 4.1.** the structures of alicyclic-β-amino-carboxylic acids (β-Acc) are shown which were used to replace D-Ala^2 in dermorphin. While the cis-β-Acc residues (I, III) introduce significant constrains into the peptide, the trans residues of the same substituents (II, IV) are conformationally more flexible. The potency of the new ligands was compared in ligand binding experiments using the universal opioid antagonist ligand, [^{3}H]naloxone in rat brain membranes (**Fig. 4.2.**). The parent peptide, dermorphin displayed the highest potency to opioid receptors, having an IC_{50} of about 50 nM in this assay. Substitution of its D-Ala^2 by the rigid β-Acc derivatives attenuated opioid binding; all of the new analogues inhibited the equilibrium binding of [^{3}H]naloxone with much lower affinity. DMF-68, -70, -63, -62 containing a bicyclic complex ring structure (cis- or trans-norbornane and norbornene, respectively) were able to displace [^{3}H]naloxone binding only at very high (100 μM) concentrations (**Fig. 4.2. right**).

Table 4.1. Structural and analytical features of the new dermorphin analogues (Tyr^1-X^2-Phe^3-Gly^4-Tyr^5-Pro^6-Ser^7-NH_2[#])

amino acid in position 2	code	calc MW	found MW	R_t
cis-2-amino-cyclohexane carboxylic acid **(I)**	DMF-58	857.1	857.8	9.51
trans-2-amino-cyclohexane carboxylic acid **(II)**	DMF-61	857.1	857.8; 856.4[*]	11.31
cis-2-amino-cyclohexene carboxylic acid **(III)**	DMF-59	855.1	855.9	11.17
trans-2-amino-cyclohexene carboxylic acid **(IV)**	DMF-60	855.1	855.8	10.95
cis-2-amino-norbornane carboxylic acid **(V)**	DMF-68	869.1	869.8	11.19
trans-2-amino-norbornane carboxylic acid **(VI)**	DMF-70	869.1	869.8	9.63
cis-2-amino-norbornene carboxylic acid **(VII)**	DMF-63	867.1	867.8	9.38
trans-2-amino-norbornene carboxylic acid **(VIII)**	DMF-62	867.1	867.9	9.68

[#] dermorphin if X: D-Ala; [*] Fab MW measurement, the others are from quadrupole electrospray spectra

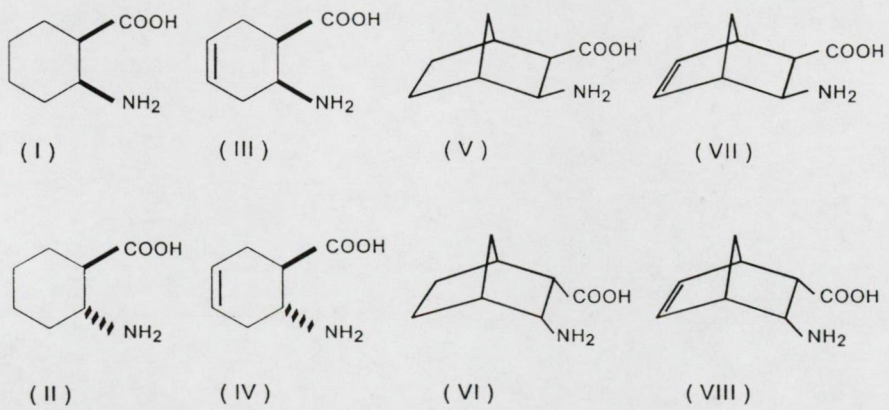

Fig. 4.1. Structure of cis (upper row) and trans (lower row β-amino-cyclic-carboxylic acids.

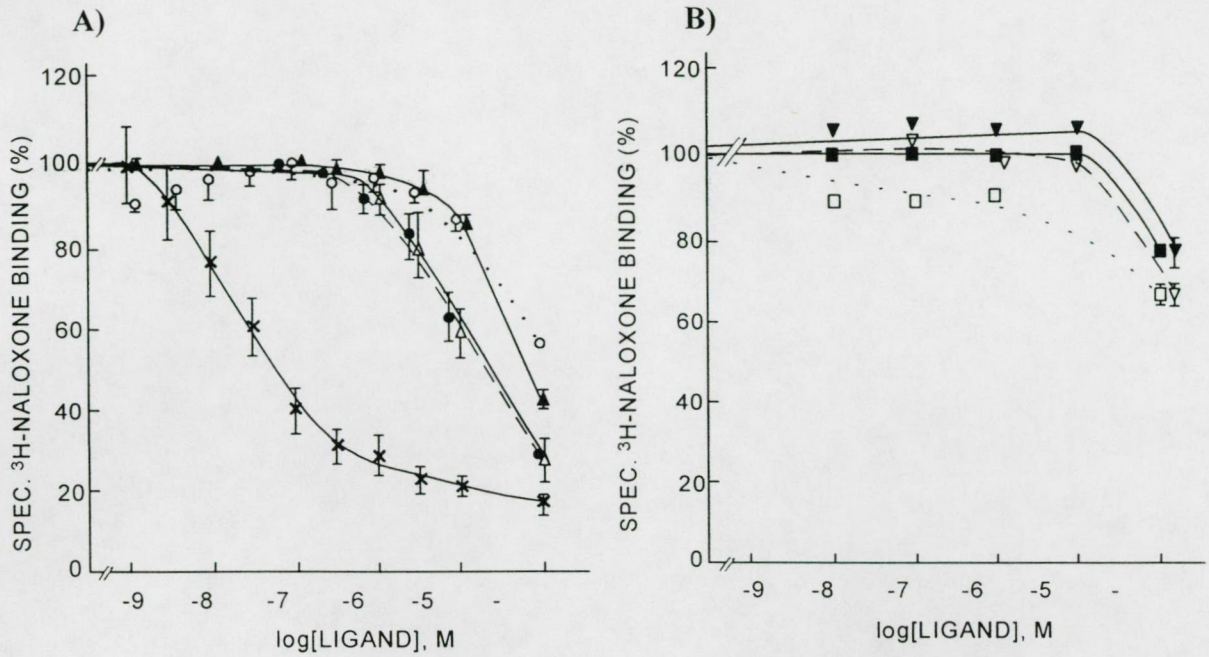

Fig. 4.2. Inhibition by dermorphin analogues of [^{3}H]naloxone specific binding to rat brain. Crude membrane fraction was incubated with [^{3}H]naloxone (1 nM) and the following unlabelled ligands DMF (×), DMF-58 (○), DMF-59 (▲), DMF-60 (Δ), DMF-61 (●), DMF-62 (□), DMF-63 (■), DMF-68 (▽), DMF-70 (▼) in 50 mM Tris-HCl (pH 7.4). Symbols represent the mean ±SEM, n ≥ 2; A); data from a single experiment, B).

4.1.2. Binding activity of DMF-60 and DMF-61 to μ- and δ-opioid receptors

The two relatively most potent new derivatives, DMF-60 and DMF-61 ([trans-β-amino-cyclohexane carboxylic acid]^2-and [trans-β-amino-cyclohexene carboxylic acid]^2 dermorphin) were further tested for their selectivity to μ- vs. δ–opioid binding (**Fig 4.3., Table 4.2.**). The substituted ligands possessed weak binding affinity (K_i in the micromolar range) to either opioid receptor types, although the loss of potency to [^{3}H]Ile^{5,6}-deltorphin sites was less than to [^{3}H]DAMGO binding sites, thus conferring some 'δ-preference' to these peptides (**Table 4.2.**). The K_i values of DAMGO and dermorphin measured in our experiments are in good agreements with literature data (Schiller P.W. 1991 and references cited therein).

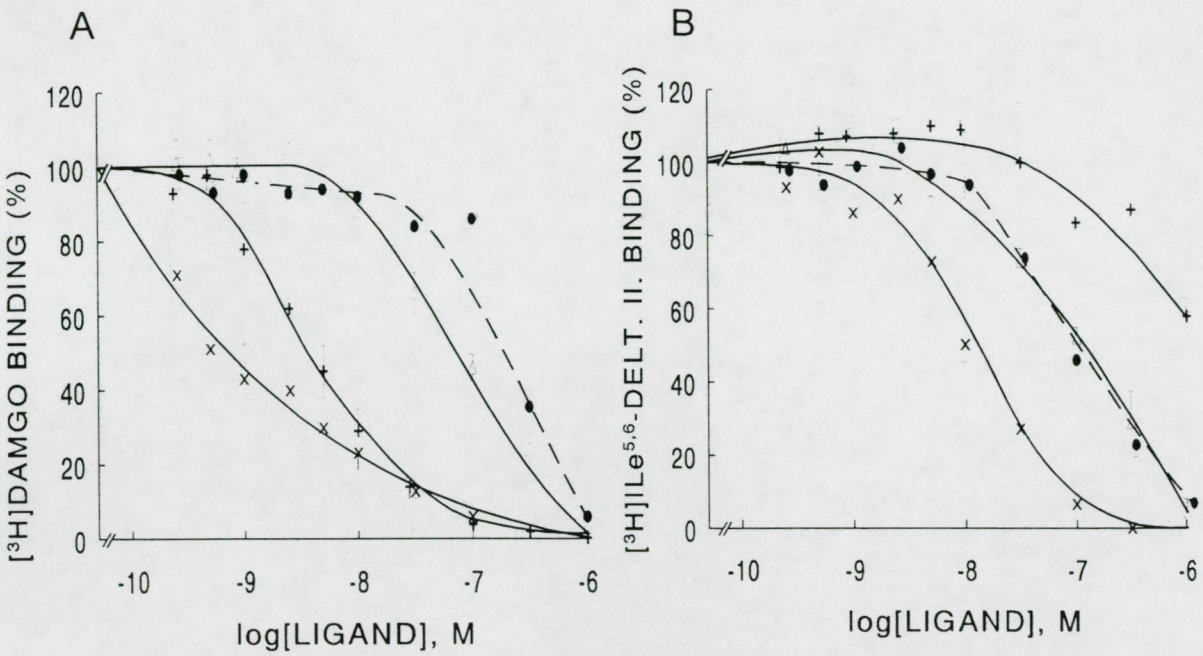

Fig. 4.3. Displacement of (A) [^{3}H]DAMGO or (B) [^{3}H]Ile^{5,6}-deltorphin II binding by various concentrations of DAMGO (+), DMF-60 (Δ), DMF-61 (•), or DMF (×) in rat brain membrane fraction. Results are expressed as percentage of control binding, i.e. binding in the absence of displacers. When error bars are smaller than the symbols, only the latter are depicted.

Table 4.2. Binding affinities of dermorphin analogues to μ– and δ–opioid receptors in rat brain

	[^{3}H] DAMGO (μ) K_i (nM)	[^{3}H]Ile^{5,6}-deltorphin II (δ) K_i (nM)	Ki (δ)/ Ki (μ)
DAMGO	3.3 ± 0.5	634 ± 150*	192
DMF	1.7 ± 0.5	158 ± 21	90
DMF-60	2200 ± 460*	1620 ± 290	0.73
DMF-61	3390 ± 1820*	1170 ± 270	0.34

n=3 except * where n=2

4.2. G-protein activation of Met^5-enkephalin-Arg^6-Phe^7 derived peptides

4.2.1. G-protein activation of MERF and two of its derivatives

Previous ligand binding experiments suggested that MERF might bind with highest affinity to the so-called κ_2-opioid receptors (Benyhe et al. 1999). This is a hypothetical receptor type that lacks specific ligands thus its pharmacological characteristics as well as signal transduction pathway is yet to be described. We have shown that the specific [^{3}H]MERF binding was inhibited in the presence of 100 μM GppNHp in frog and rat brain membranes suggesting that these binding sites might be working through heterotrimeric G-proteins (Bozó et al. 2000). Recently, Rottmann et al. (1998) described the G-protein types and their activation by κ-opioid ligands in frog brain membranes.

In order to corroborate on this finding, the G-protein activation of MERF and its analogues was examined with the ligand stimulated [^{35}S]GTPγS binding assay. This recently developed fuctional assay reflects the GDP-GTP exchange on G_α when the receptor is activated by agonists (Sim et al. 1995; Traynor et al. 1995).

As described in chapter 1.4.2. the assay conditions, particularly the concentration of GDP, are crucial for obtaining proper signal to noise ratio. Therefore [^{35}S]GTPγS binding was investigated in the presence of various concentrations of GDP, in the absence and presence of a maximally effective concentration (10^{-5} M) of the μ-selective agonist DAMGO. [^{35}S]GTPγS binding was decreased by increasing concentrations of GDP both in the presence and absence of agonist. However the effect of GDP was more pronounced on the basal activity (**Fig 4.4.A**). Detectable stimulation by DAMGO could only be observed in the presence of micromolar concentrations of GDP. The percent stimulation of [^{35}S]GTPγS binding by agonist was increased by increasing concentration of GDP, up to a maximal value of 44 % at 100 μM GDP (**Fig. 4.4.B.**). Therefore, further experiments were performed in the presence of 100 μM GDP to afford an optimal signal-to-noise ratio.

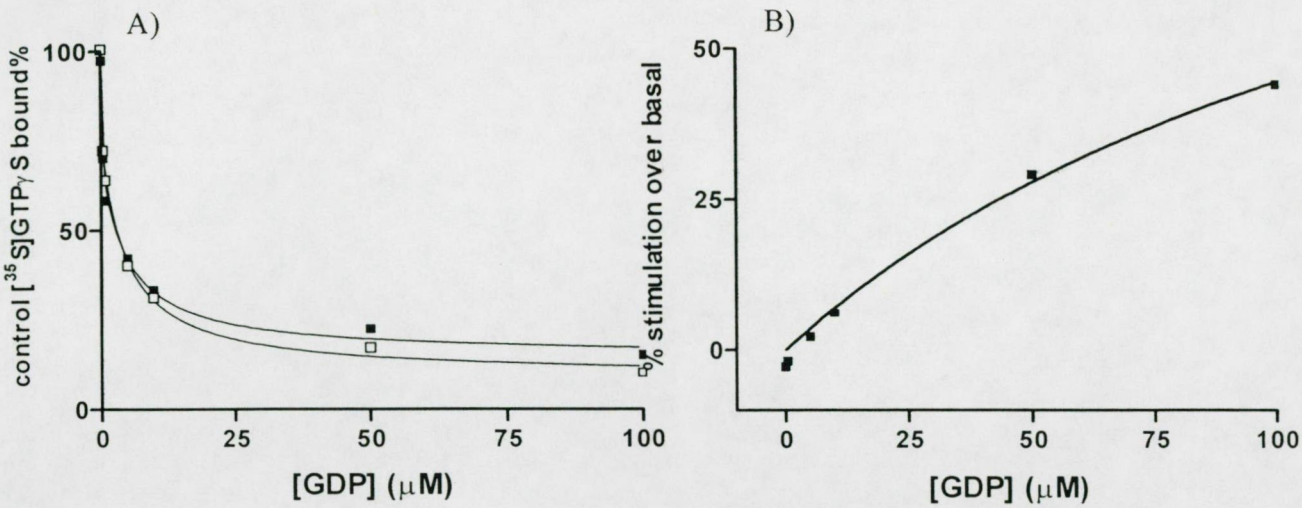

Fig. 4.4. Effect of GDP on basal and DAMGO stimulated [^{35}S]GTPγS binding in rat brain crude membrane fraction. A) GDP concentration-effect curve of DAMGO stimulated G-protein activity. Membranes were incubated with various concentrations of GDP in the absence (□) and presence (■) of 10^{-5} M DAMGO. Data represent percentage total [^{35}S]GTPγS binding measured in the absence of GDP or DAMGO. B) DAMGO-stimulated [^{35}S]GTPγS binding expressed as percentage basal [^{35}S]GTPγS binding at each concentration of GDP.

Two synthetic analogues of MERF with single or double amino acid substitutions were prepared by solid phase peptide synthesis to achieve proteolytically more stable structures: Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I),** Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe **(II).**

Detectable stimulation of [^{35}S]GTPγS binding could only be measured in the absence of protease inhibitors which were, however, necessary for the binding experiments (Benyhe et al. 1997). Integrity of the peptides was therefore checked by HPLC after the incubation with brain membranes. No significant degradation of the peptides was detected at the end of 60 min incubation at 30°C with either tissues preparations under the conditions of [^{35}S]GTPγS binding measurements. Typically, the peptides displayed more then 98% recovery in the chromatographic run.

Table 4.3. Potency (EC_{50}) and maximal effects of MERF and its derivatives in stimulating [^{35}S]GTPγS binding in frog and rat brain membranes.

Peptide	EC_{50} (nM)		Maximal stimulation (%)	
	frog	rat	frog	rat
MERF	6.7 ± 1.2	21 ± 10	116 ± 1.4	99 ± 5.8
Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I)**	9.8 ± 4	24 ± 16	81.6 ± 0.2	105 ± 2.1
Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe **(II)**	> 1000	160 ± 14	—	59 ± 6

Agonist stimulated [^{35}S]GTPγS binding was determined as described in Materials and Methods. Basal activities measured in the absence of opioid ligands were 70.6 ± 6 fmol/mg protein and 26 ± 2 fmol/mg protein in frog and rat brain membranes, respectively.

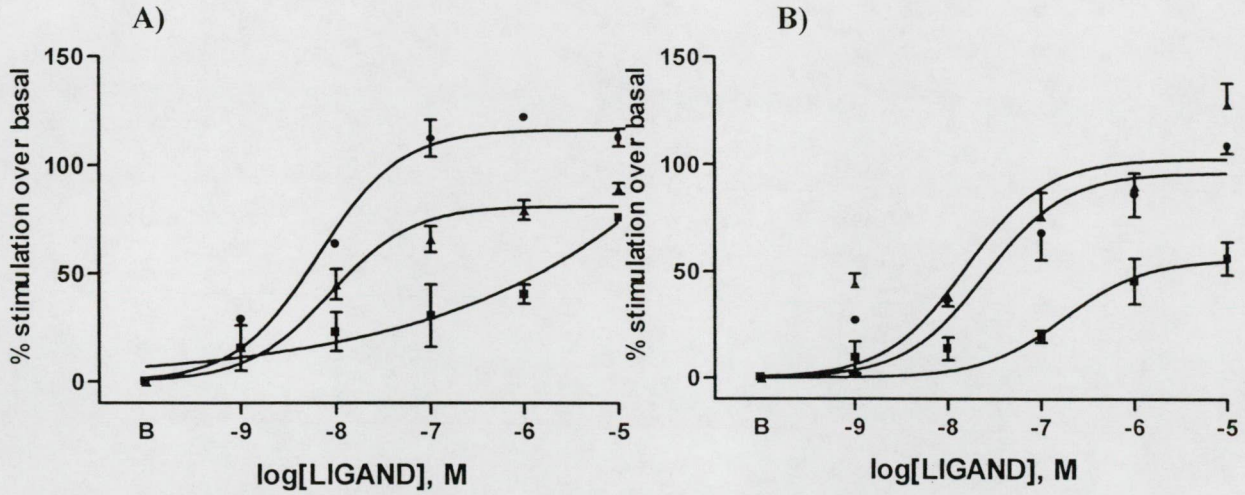

Fig.4.5. Effect of MERF and its derivatives on [^{35}S]GTPγS binding in frog (A) and rat (B) brain membranes. Incubation was performed as described in Materials and Methods. MERF (●), Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I)** (▲), Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe **(II)** (■). Data are mean ± S.E.M. of three experiments each performed in triplicate. Data are fitted with Graph Pad Prism 2.01.

The effect of the peptides was examined using increasing concentrations (10^{-9}-10^{-5}M) of MERF and its derivatives in frog and rat brain membranes (**Fig. 4.5.**). All of the peptides investigated resulted in a concentration-dependent stimulation of [^{35}S]GTPγS binding, which implied that these receptors functionally couple to G-proteins (**Fig. 4.5.**). The resulting EC_{50} (concentration producing 50% stimulation) and maximal stimulation values are summarized in **Table 4.3.**. Analog **(II)** stimulates [^{35}S]GTPγS binding only by 59% over the basal activity in rat brain membranes (**Table 4.3.**). Analog **(I)** displayed similar maximal stimulation to MERF (105 ± 2.1% and 99 ± 5.8%, respectively) in rat but lower than MERF (81.6 ± 0.2 and 116 ± 1.4, respectively) in frog brain membranes (**Table 4.3.**; **Fig. 4.5.**). EC_{50} values of analog **(II)** were the highest, showing the lowest potency in both tissues (**Table 4.3.**).

4.2.2. Inhibition of agonist stimulated [^{35}S]GTPγS *binding by opioid receptor antagonists*

Previous studies revealed that MERF binds to κ_2- and δ-opioid binding sites, as well as to non-opioid sites, but no binding to κ_1-opioid receptors could be detected in rat and frog brain membranes (Wollemann et al. 1994; Benyhe et al. 1997). The receptor specificity of the peptides was further investigated by measuring the inhibition of agonist stimulated [^{35}S]GTPγS binding by μ-, δ- and κ-opioid selective antagonists. Norbinaltorphimine (nor-BNI), a κ-receptor selective antagonist displayed the highest potency in inhibiting the effects of MERF or its analog. Nevertheless, the δ-opioid antagonist naltrindole was also very potent in both tissues. The μ-selective antagonist cyprodime was the least effective in both tissues (**Fig. 4.6.**). The IC_{50} values (concentration producing 50% inhibition of the maximal inhibition of the ligand) of the antagonists were significantly lower in frog than in rat brain (**Table 4.4.**).

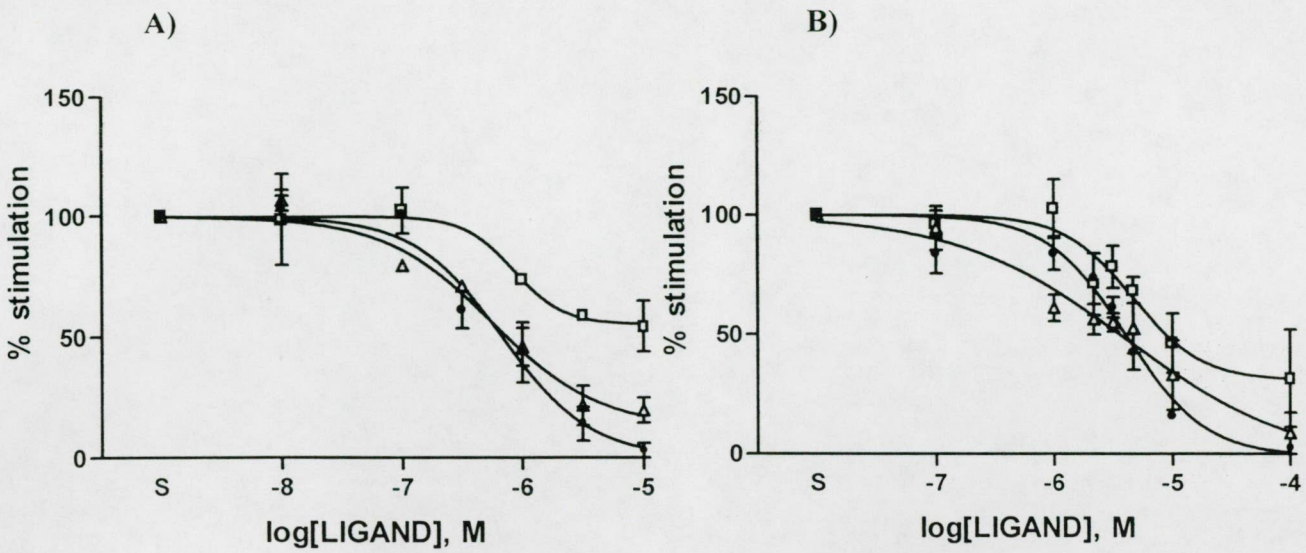

Fig. 4.6. Inhibition of ligand (MERF and Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I)** in frog and rat brain membranes, respectively) stimulated [^{35}S]GTPγS binding by antagonists - Nor-BNI (●), naltrindole (Δ) and cyprodime (□) - in frog (A) and rat (B) brain membranes. S: stimulation in the absence of antagonists. Incubation and filtration were performed as described in Materials and Methods. Data are mean ± S.E.M. of three experiments each performed in triplicate. Data were fitted with Graph Pad Prism 2.01.

Table 4.4. Effect of site-specific opioid receptor antagonists on agonists stimulated [^{35}S]GTPγS binding in frog and rat brain membranes.

Selective antagonists	IC_{50} (μM)	
	frog brain	rat brain
Norbinaltorphimine (κ)	0.67 ± 0.01	4.0 ± 0.09
Naltrindole (δ)	0.56 ± 0.01	4.1 ±0.32
Cyprodime (μ)	0.85 ± 0.02	4.6 ± 0.15

4.3. Regulatory changes induced by μ-opioid agonist treatments in rat brain

In the present work the regulatory changes induced by various μ-opioid agonist treatments were examined in rat brain. Brains exposed to agonists *in vivo* and control rats treated the same way with the appropriate solvent substituting for agonists were simultaneously assessed in every experiment. Control and treated brain homogenates were subjected to subcellular fractionation to prepare highly purified synaptic plasmamembranes (SPM) and 'light vesicle' (LM) fractions.

To assess changes in the intracellular distribution of μ-opioid receptors, subcellular fractionation was combined with radioligand binding measurements. The degree of desensitization was estimated by ligand-stimulated [^{35}S]GTPγS functional assays.

4.3.1. Radioligand binding to μ-receptors in subcellular membrane fractions after drug treatment

Rat were given an acute injection of *etorphine* at 0.4 mg/kg as published by Keith et al. (1998). These authors detected rapid internalization of μ-opioid receptors by combining immunofluorescence with confocal microscopy (Keith et al. 1998).

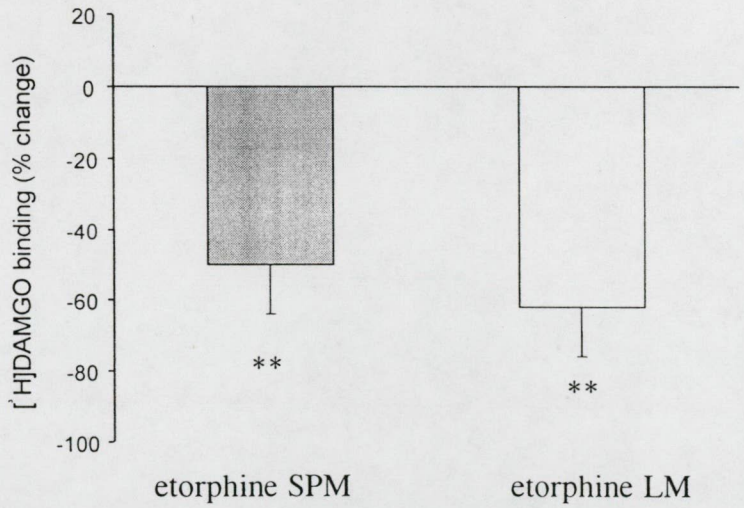

Fig. 4.10. Changes in the B_{max} of [^{3}H]DAMGO binding due to **etorphine** exposure in rat brain subcellular fractions (SPM, LM). Results shown are expressed as % changes due to agonist treatments in etorphine SPM; etorphine LM compared to matched control values in vehicle treated fractions (not shown). Mean ± S.E.M., n = 3-8. **p< 0.05.

We showed that the number of μ-binding sites decreased by 50 respectively 62% in SPM and LM after high dose of etorphine exposure (**Fig. 4.10., Table 4.5.**). The K_d values were 1.93 ± 0.49 nM (SPM) and 2.43 ± 0.21 nM (LM) in control animals and 3.45 ± 1.14 nM (SPM) and 1.5 ± 0.23 nM (LM) in etorphine treated samples (**Table 4.5.**).

Rats were chronically treated with *morphine* that elicited significant analgesic tolerance measured in hot plate and tail withdrawal assays (Fábián et al., in preparation). Agonist-induced changes in the ligand binding activity of μ-opioid receptors were analysed with [^{3}H]DAMGO homologous displacement experiments. In control animals, the K_d was 2.65 ± 0.30 nM, B_{MAX} was 203 ± 29 fmol/mg protein in SPM. These values were 3.00 ± 0.3 nM and 200 ± 17 fmol/mg protein in LM, respectively. These data are in good agreements with previous results (Szűcs et al. 1992). Chronic morphine treatment caused no significant change in the number of surface (SPM) μ-binding sites. In contrast, the B_{MAX} of [^{3}H]DAMGO binding was elevated by 65% in the LM of morphine treated animals (**Fig. 4.11. Table 4.5.**).

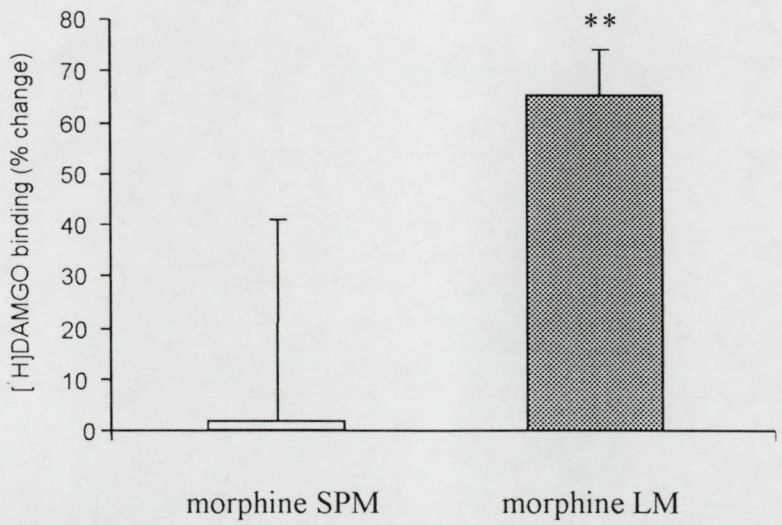

Fig. 4.11. Changes in the B_{max} of [^{3}H]DAMGO binding due to chronic **morphine** exposure in rat brain subcellular fractions (SPM, LM). Results shown are expressed as % changes due to agonist treatments in morphine SPM; morphine LM compared to matched control values in vehicle treated fractions (not shown). Mean ± S.E.M., n = 3-8. **p< 0.05.

Rats were also rendered tolerant by the μ-opioid specific peptide *DAMGO* and its chloromethyl ketone derivative *DAMCK*. The peptides were injected intracerebroventricularly for 5 days at 100 ng/kg once daily ('low dose') and for 8 days at 10 μg/kg twice daily ('high dose').

After chronic DAMCK treatment, the number of μ-opioid receptors decreased by ≈ 28% in SPMs (**Fig. 4.12.A**). The decrease measured after DAMGO treatment in SPM has only reached a statistically significant extent after 'high-dose' treatment (**Fig. 4.12.B**). The number of [^{3}H]DAMGO binding sites was elevated in LM after both 'low-' and 'high-dose' treatment with either DAMGO or DAMCK (**Fig. 4.12.A-B**).

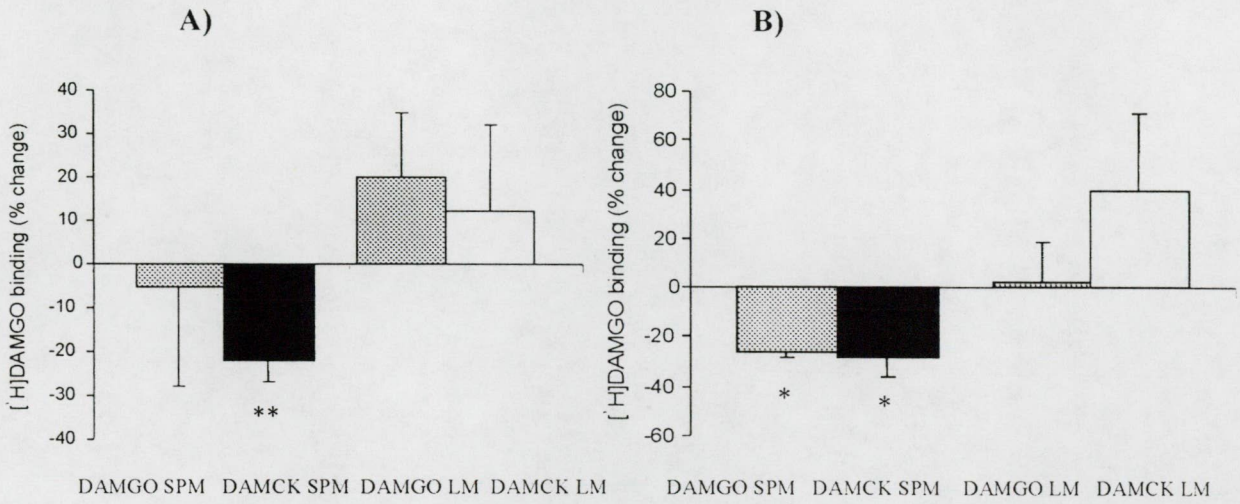

Fig. 4.12.A-B Changes in the B_{max} of [^{3}H]DAMGO binding due to chronic **DAMGO** and **DAMCK** at 'low-dose' A), or chronic **DAMGO** and **DAMCK** at 'high-dose' B) exposure in rat brain subcellular fractions (SPM, LM). Results shown are expressed as % changes due to agonist treatments in DAMGO SPM; DAMCK SPM; DAMGO LM; DAMCK LM compared to matched control values in vehicle treated fractions (not shown). Mean ± S.E.M., n = 3-8. *P< 0.1, **p< 0.05.

Table 4.5. Changes in [^{3}H]DAMGO binding induced by in vivo opioid exposure in rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions.

Membrane	fractions	K_d nM	B_{max} fmol/mg protein	B_{max} % control[a]
SPM	control	1.93 ± 0.49	205 ± 24	
	etorphine	3.45 ± 1.14	124 ± 52	**50 ± 14****
LM	control	2.43 ± 0.21	167 ± 15	
	etorphine	1.50 ± 0.23	**63 ± 18****	**38 ± 12****
SPM	control	2.65 ± 0.30	203 ± 29	
	morphine	2.60 ± 0.20	187 ± 82	101 ± 40
LM	control	3.00 ± 0.30	200 ± 17	
	morphine	4.00 ± 0.40	301 ± 50	**165 ± 9****
	control	1.50 ± 0.23	167 ± 15	
SPM	DAMGO (l.d.)	1.21 ± 0.05	147 ± 21	95 ± 23
	DAMCK (l.d.)	1.52 ± 0.31	141 ± 14	**78 ± 5****
	control	2.29 ± 0.28	181 ± 24	
LM	DAMGO (l.d.)	2.02 ± 0.45	210 ± 45	120 ± 15
	DAMCK (l.d.)	2.38 ± 0.31	**225 ± 3***	112 ± 20
	control	1.54 ± 0.26	130 ± 15	
SPM	DAMGO (h.d.)	1.36 ± 0.23	104 ± 25	**78 ± 5***
	DAMCK (h.d.)	1.11 ± 0.27	**81 ± 13****	**72 ± 8***
	control	1.61 ± 0.14	130 ± 22	
LM	DAMGO (h.d.)	1.97 ± 0.39	125 ± 41	102 ± 17
	DAMCK (h.d.)	2.24 ± 1.25	192 ± 19	140 ± 31

Results are mean ± S.E.M. of n = 3-8 experiments. *P< 0.1,
** p < 0.05. [a] Matched samples of treated vs. control.
(l.d.): 'low dose' treatment
(h.d.): 'high dose' treatment

4.3.2. Agonist-stimulated [^{35}S]GTPγS binding in subcellular membrane fractions after drug treatment

Functional coupling of μ-opioid receptors to G-proteins before and after opioid agonist treatments was examined by measuring the ability of DAMGO added to membrane fractions *in vitro* to stimulate [^{35}S]GTPγS binding. DAMGO resulted in a concent+ration-dependent stimulation of [^{35}S]GTPγS binding that was inhibited by the opioid antagonist naloxone (100 μM) implying that the effect of DAMGO was due to activation of opioid receptors (data not shown).

Etorphine treatment resulted in a significant shift to the right of the dose-response curve of DAMGO stimulated [^{35}S]GTPγS binding and led to increased EC_{50} values from 62 ± 2 to 358 ± 11 nM in SPM. There was an even higher extent of increase in the EC_{50} of DAMGO stimulation in LM (**Fig. 4.13., Table 4.6.**).

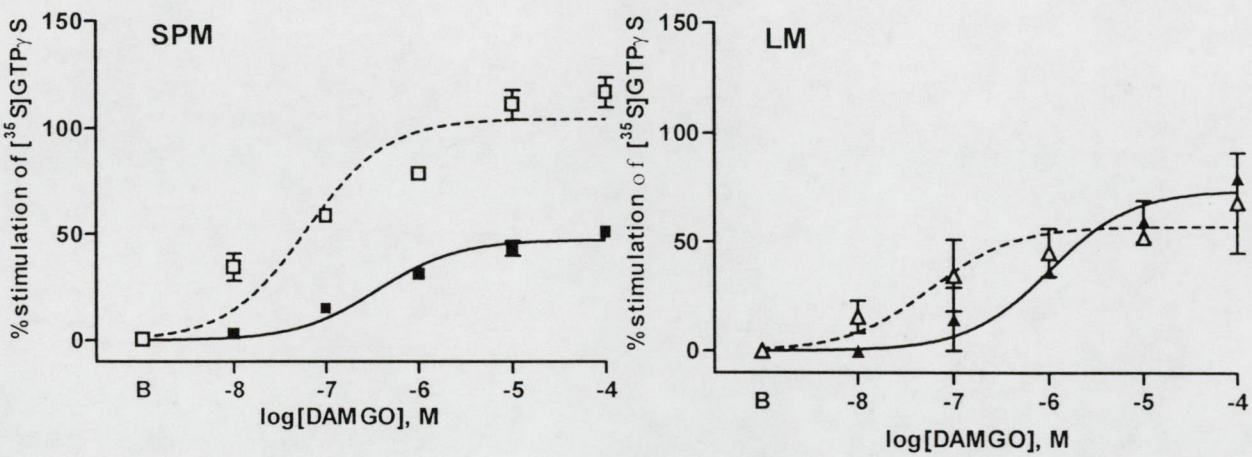

Fig. 4.13. Stimulation of [^{35}S]GTPγS binding by different concentrations of DAMGO in control and **etorphine** treated (0.4 mg/kg) rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions. Control SPM (□), control LM (Δ), treated SPM (■), treated LM (▲). Data are mean ± S.E.M. of 2 experiments each performed in triplicate. Data were fitted with Graph Pad Prism 2.01.

The maximal effect of DAMGO in stimulating [^{35}S]GTPγS binding slightly decreased, but the EC_{50} value of DAMGO has not significantly changed in the SPM after chronic *morphine* treatment (**Fig. 4.14., Table 4.6.**). An opposite phenomenon, namely a leftward shift of the dose-response curve of DAMGO was detected in morphine treated LM (**Fig 4.14.**).

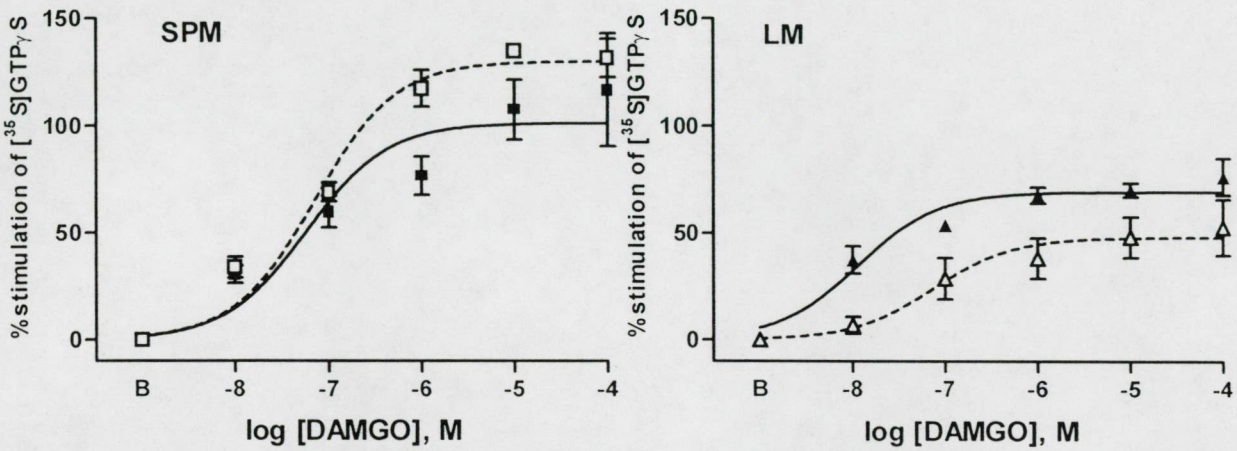

Fig. 4.14. Stimulation of [^{35}S]GTPγS binding by different concentrations of DAMGO in control and chronic **morphine** treated rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions. Control SPM (□), control LM (Δ), treated SPM (■), treated LM (▲). Data are mean ± S.E.M. of 3-6 experiments each performed in triplicate. Data were fitted with Graph Pad Prism 2.01.

No significant changes were measured in the EC_{50} values of DAMGO stimulated [^{35}S]GTPγS binding after either dose of *DAMGO* or *DAMCK* treatment in the SPM fractions (**Table 4.6., Fig. 4.15.; 4.16.**).

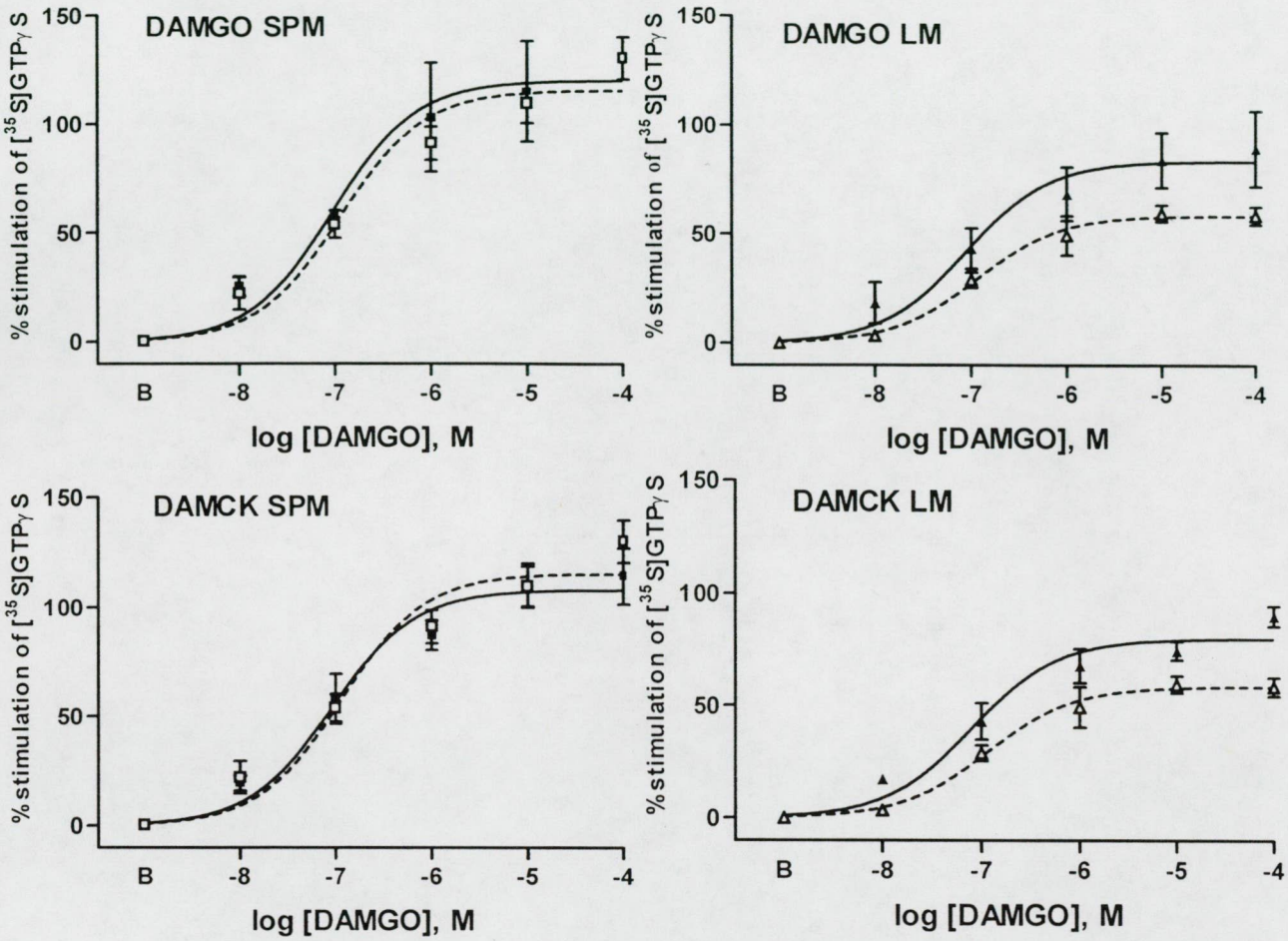

Fig. 4.15. Stimulation of [^{35}S]GTPγS binding by different concentrations of DAMGO in control and chronic **DAMGO** treated (**'low dose'**); control and chronic **DAMCK** treated (**'low dose'**) rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions. Control SPM (□), control LM (Δ), treated SPM (■), treated LM (▲). Data are mean ± S.E.M. of 2 experiments each performed in triplicate. Data were fitted with Graph Pad Prism 2.01.

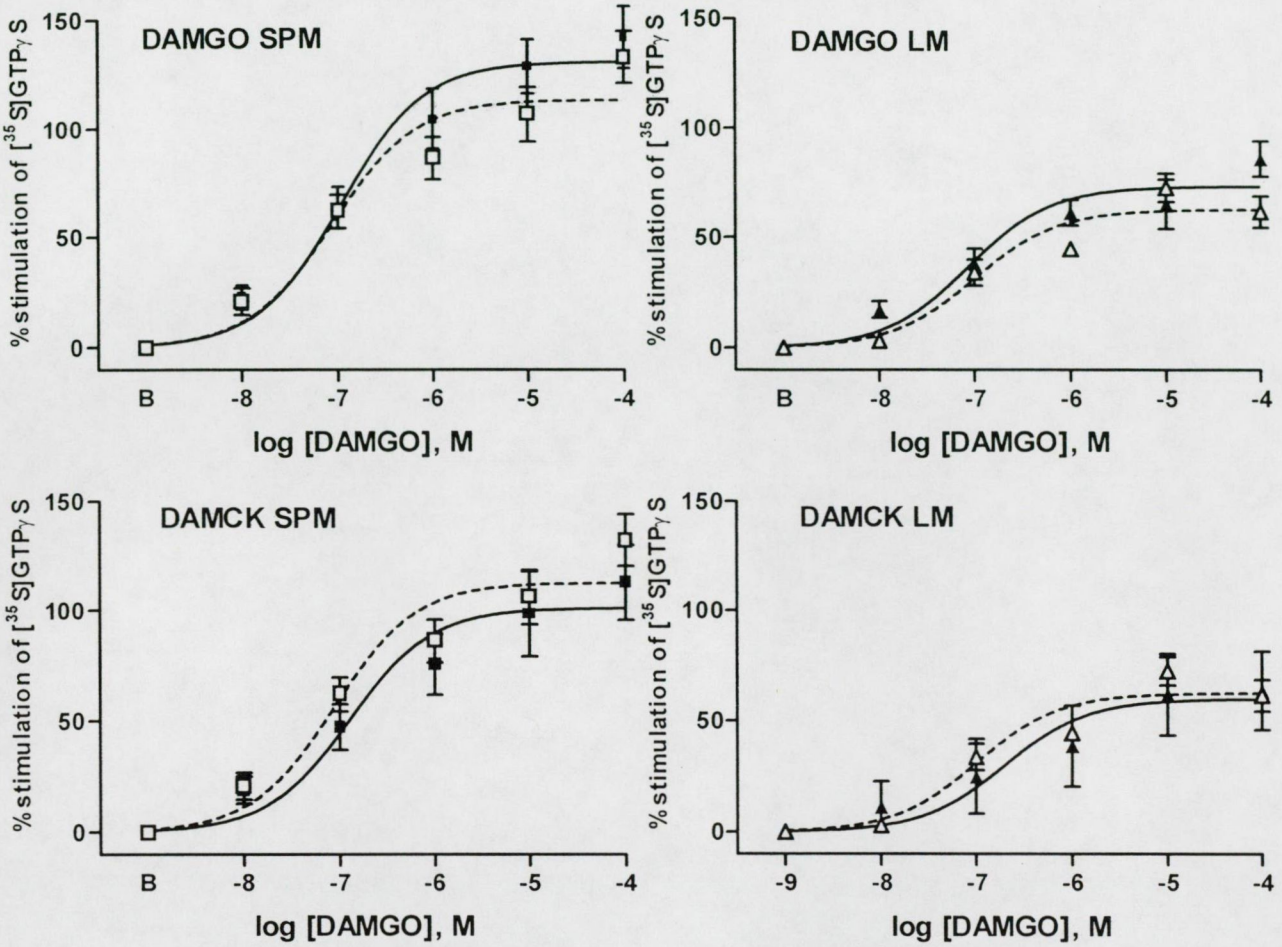

Fig. 4.16. Stimulation of [^{35}S]GTPγS binding by different concentrations of DAMGO in control and chronic **DAMGO** treated (**'high dose'**); control and chronic **DAMCK** treated (**'high dose'**) rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions. Control SPM (□), control LM (Δ), treated SPM (■), treated LM (▲). Data are mean ± S.E.M. of 2 experiments each performed in triplicate. Data were fitted with Graph Pad Prism 2.01.

'Low-dose' (100 ng) treatment significantly elevated the maximal stimulation by DAMGO of [^{35}S]GTPγS binding from 59 ± 4% in vehicle treated LM to 82 ± 16 and 81 ± 8% in LM fractions of DAMGO and DAMCK treated brains, respectively (**Table 4.6., Fig. 4.15**). Similar changes were not detected after 'high-dose' exposure to either DAMGO or DAMCK.

Table 4.6. Changes in DAMGO stimulated [^{35}S]GTPγS binding induced by in vivo opioid exposure in rat brain synaptic plasmamembrane (SPM) and light vesicular (LM) fractions.

Membrane fractions		EC_{50} (nM)	Max. effect (%)	Basal activity (fmol/mg)
SPM	control	**62 ± 2**	**104 ± 1**	36 ± 3
	etorphine	**358 ± 11****	**47 ± 3****	45 ± 14
LM	control	**57 ± 3**	60 ± 19	28 ± 3
	etorphine	**1009 ± 173****	74 ± 4	27 ± 9
SPM	control	70 ± 8	127 ± 5	50 ± 11
	morphine	57 ± 2	96 ± 20	61 ± 9
LM	control	**73 ± 3**	**49 ± 1**	39 ± 10
	morphine	**11 ± 1****	**67 ± 3****	27 ± 4
SPM	control	115 ± 11	116 ± 8	87 ± 13
	DAMGO (l.d.)	94 ± 10	126 ± 14	110 ± 32
	DAMCK (l.d.)	90 ± 10	107 ± 12	126 ± 29
LM	control	113 ± 13	**59 ± 4**	53 ± 8
	DAMGO (l.d.)	87 ± 12	**82 ± 16****	53 ± 12
	DAMCK (l.d.)	79 ± 12	**81 ± 8****	76 ± 21
SPM	control	87 ± 9	112 ± 12	70 ± 10
	DAMGO (h.d.)	107 ± 14	130 ± 14	60 ± 3
	DAMCK (h.d.)	126 ± 22	100 ± 9	89 ± 33
LM	control	**101 ± 14**	60 ± 3	42 ± 6
	DAMGO (h.d.)	86 ± 2	72 ± 9	42 ± 9
	DAMCK (h.d.)	**200 ± 42****	61 ± 17	34 ± 7

** denotes those data where the treated are significantly different from the control. **P< 0.05. Data are mean ± S.E.M. of 3-6 experiments each performed in triplicate.

(l.d.): 'low dose' treatment

(h.d.): 'high dose' treatment

5. DISCUSSION

Opioid ligands play a crucial role in controlling the nociceptive pathways via μ-, δ– and κ–opioid receptors. In this study the three main characteristics of the opioid agonists (affinities and selctiviy for the opioid receptor types, G-protein activation and agonist-induced receptor regulatory mechanisms) have been investigated.

I. In the first part of the study binding activity of synthetic dermorphin analogues to opioid receptors was tested in rat brain membrane fractions. Our results showed that introduction of rigid cyclic β-amino-carboxylic acids in the 2nd positions of the peptide backbone resulted in different effects for dermorphins than for casomorphins (Mierke et al. 1990). Here the rigid cyclohexane, norbornane and their unsaturated analogues highly decreased the binding potency of the resulting compounds to μ- and δ–opioid receptors. In contrast, Pro^2 or other residues causing constrained topology are crucial for proper orientation of Tyr and Phe, thus for the biological activity of morphiceptin (Mierke et al. 1990). It was previously reported that introduction of D-Ala^2 into morphiceptin leads to an enhancement of the binding affinity to δ-sites, thus resulting in a reduced μ-selectivity (Brantl et al. 1981; Liebmann et al. 1986). Therefore we speculate that interaction of the larger heptapeptide derivatives of dermorphins with the μ-receptor is distinct from that of the tetrapeptide morphiceptin. Indeed, the K_i value for the former was found to be 0.33 nM, while 56 nM was reported for morphiceptin against the cloned μ-opioid receptor by Raynor et al. (1994). It cannot be excluded that these ligands would bind to different subtypes of μ-receptor, i.e. to the yet hypothetical μ_1- and μ_2-receptors (Pasternak, 1986) which might be distinct proteins, or different binding regions of the same protein. The latter was recently demonstrated to be the case with cloned kappa receptors (Mansour et al. 1995). Although such distinction still awaits in the case of the μ-receptor, results of the present study describing the binding characteristics of constrained dermorphin derivatives contribute to the understanding of the structural and topographical requirements of peptide ligand binding.

Approaches to design new, conformationally constrained peptides are currently of great interest because of the importance of achieving highly site-specific ligands that are required for the elucidation of the structural requirements and physiological role of the opioid binding sites.

II. In the second part of the study the G-protein activation of MERF and its synthetic analogues was examined in frog and rat brain crude membrane fractions.

Cloned κ-, δ- and μ-receptors interact with multiple G-proteins (Chakrabarti et al. 1995; Prather et al. 1994; Prather et al. 1995). There are several reports in the literature on functionally G-protein coupled μ- and δ-opioid receptors in mammalian brain membranes (Selley et al. 1998; Sim et al. 1998). However, little data are available on the signalling pathways, including the features of G-protein activation, of endogenously expressed κ-opioid receptors, especially in frog brain membranes.

Our experiments were performed at high GDP concentration, which was required to demonstrate differences in the relative efficacies (Breivogel et al. 1998).

The EC_{50} values of analog **(II)** were the highest in frog and rat brain membranes, which refers to the lowest potency of this ligand. Analog **(II)** seemed to be the lest efficacious peptide, showing only 59% stimulation of [^{35}S]GTPγS binding. These results are in a good agreement with those obtained in the ligand binding experiments. As shown by Benyhe et al., synthetic MERF derivative **(I)** inhibited the equilibrium binding of [^{3}H]MERF with relatively high affinity in frog and rat brain membrane fractions. The rank order of the potency obtained in homologous and heterologous displacement experiments was similar in both tissues: MERF > Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe **(I)** >> Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe **(II)** (Bozó et al. 2000). It was suggested that replacement of Met^{5} with D-Nle^{5} markedly decreases the affinity of derivative (II). Significant increases of the IC_{50} values in the presence of sodium ions suggested the agonist character of all the peptides tested (Bozó et al. 2000).

Analog **(I)** showed lower maximal stimulation than MERF in frog but similar to MERF in rat brain membranes which might be due to differences in the receptor specificity of the two peptides.

The κ-selective antagonist norbinaltorphimine and the δ-selective antagonist naltrindole displayed similar potency in inhibiting the effects of the peptides. The μ-selective antagonist cyprodime seemed to be the least potent. Thus it can be deduced that the effects of MERF and analog **(I)** are mediated the least by μ-opioid receptors. These results agree with the receptor binding experiments using [^{3}H]DAMGO (data not shown).

The IC_{50} values of the antagonists were significantly higher in rat than in frog brain membranes (**Table 4.4.**). This is in a good agreement with previous results obtained from ligand binding experiments in the two preparations and might be explained by the different

characters of the receptors present in the two tissues (Wollemann et al. 1993; Wollemann et al. 1994). Opioid antagonists seemed to be more effective in inhibiting the effects of the agonists in frog brain membranes suggesting their higher affinity to these binding sites. Indeed, ligand affinities, stereoselectivity and the proportions of opioid receptor types have been reported to be characteristically different in amphibian brain (Benyhe et al. 1990; Benyhe et al. 1999; Simon et al. 1984), allowing to designate the major type of opioid receptors in frog as κ_2-receptor (Wollemann et al. 1993). These data are in agreement with IC_{50} values obtained from the heterologous displacement curves using [^{3}H]MERF and various antagonists in frog and rat brain membranes (Benyhe et al. 1997; Wollemann et al. 1994). Other investigators termed this particular receptor-type either as 'op'-receptor (Mollereau et al. 1988), or more recently the 'unireceptor' term has been introduced (Stevens and Newman, 1999).

As previously shown by Jordan et al. (1999) and Slowe et al. (1999) there may be functional interaction between μ- and δ-receptors and κ_1- and δ-receptors. The heterodimerization of two fully functional opioid receptors results in a new receptor that is distinct from those of either receptor and binds highly selective agonists and antagonists with much lower affinity than non-selectice ligands. It is possible that the hypothetical κ_2-receptors may be such κ_1- and δ-receptor dimers.

Summarizing these results, MERF-releted peptides are able to activate G-proteins via opioid receptors. The substitution of Met^5 by D-Nle^5 leads to a decrease of the potency of analog **(II)**. MERF and analog **(I)** have no distinctive selectivity to any of the opioid receptor types in frog and rat brain crude membrane fractions, at least under the conditions of [^{35}S]GTPγS binding measurements. Some of these peptides are selected for radiolabelling and will be used in future experiments investigating multiple MERF binding sites in the central nervous system.

III. In the third part of the study the changes in the ligand binding parameters of μ-opioid receptors and the receptor G-protein interaction were analysed after agonist treatment using [^{3}H]DAMGO homologous displacement experiments and ligand stimulated [^{35}S]GTPγS binding assays in subcellular fractions of rat brains. The K_d values were not significantly changed due to agonists treatments (**Table 4.5.**) showing that the procedure yielding subcellular fractions effectively removed residual exogenous opioids. In the case of etorphine tratment additional washing steps in the presence of GTP and NaCl were necessary

as described in Materials and Methods. There were comparable numbers of [^{3}H]DAMGO binding sites in vehicle-treated control SPM and control LM fractions (**Table 4.5.**). However, agonist exposure elicited notable ligand-specific changes in the subcellular distribution of μ-sites as shown in **Table 4.5.** and outlined below.

Etorphine was injected at 0.4 mg/kg which dose had been shown previously to induce rapid internalization of μ-opioid receptors with a decrease of receptor immunoreactivity in the plasma membrane and concomitant increase in intracellular vesicles (Keith et al. 1998). Our results presented here confirm and extend those findings. We demonstrate that this high dose of etorphine has led to a decrease in the number of μ-binding sites by 50 respectively 62% in etorphine treated SPM and LM (**Table 4.5.**). Thus similar results, namely internalization of μ-opioid receptors, were noted with both immunofluorescence (Keith et al. 1998) and our approach that combined subcellular fractionation with radioligand binding studies with a μ-specific radioligand. Results obtained with binding techniques were often confirmed using immunofluorescence or fluorescence methods methods (Li et al. 1999 and references cited therein) showing that either approach is suitable to study changes in receptor distribution.

When measuring DAMGO-stimulated [^{35}S]GTPγS binding, the EC_{50} values of DAMGO have been significantly elevated in etorphine-treated SPM and also in LM fractions (**Table 4.6.**). These findings indicate that the functional coupling between μ-opioid receptors and G-proteins was attenuated due to etorphine treatment (Szűcs et al., submitted). Altogether, the results of ligand binding and functional assays point to cellular changes of receptor desensitization and internalization.

When *morphine* was administered chronically, the number of surface [^{3}H]DAMGO binding sites has not changed significantly compared to that in control animals (**Table 4.5.**). These results indicated that sustained morphine exposure did not provoke detectable internalization of the surface μ-opioid receptors.

The EC_{50} of DAMGO decreased from 73 ± 3 nM to 11 ± 1 nM in LM of morphine-tolerant animals, indicating the increased coupling of the receptors to G-proteins, (**Table 4.6.**). In parallel, the extent of maximal stimulation by DAMGO increased by 37% in the LM due to chronic morphine exposure (**Table 4.6.**). This might reflect an increased number of μ-binding sites that is in a good agreement with the results of the ligand binding experiments

where a 65% up-regulation of [^{3}H]DAMGO binding sites was measured in LM after morphine treatment (**Table 4.5.**).

Taken together, these results with morphine do not support the hypothesis that suggests that surface receptors should be desensitized and/or internalized after exposure to agonists. Rather, we only detected significant changes for the intracellular [^{3}H]DAMGO binding sites that became up-regulated and more coupled in morphine tolerant animals (**Table 4.5.**). A similar phenomenon, namely up-regulation of intracellular nicotinic acethylcholine receptors (nAChR) was also reported after chronic nicotine treatment in primary cultures of fetal rat brain. It was suggested to entail a nicotine-stimulated conversion of the low-affinity reserve pool of nAChR into a high-affinity conformer (Bencherif et al. 1995; Davila-Garcia et al. 1999). It is speculated that a similar mechanism is responsible for the observed effect of morphine in subcellular fractions of rat brain. Details on the effect of chronic morphine on μ-opioid receptors and G-proteins will be published by Fábián et al (in preparation). Recent data with recombinant receptors expressed in a variety of host cells also showed no internalization with morphine (Burford et al., 1998). This intriguing phenomenon is under intensive investigations in several laboratories and might explain the unique addictive properties of morphine.

The tendency of the molecular changes examined were similar after both *DAMGO* and *DAMCK* treatments (**Table 4.5.-6.**).

Upon chronic administration of DAMGO or DAMCK either with the 'low-dose' or the 'high-dose' treatment, the B_{max} of [^{3}H]DAMGO binding decreased in SPMs by a maximal value of 28% (**Table 4.5.**). There was a concomitant increase in the number of [^{3}H]DAMGO binding sites that however has not reached a statistically significant extent (**Table 4.5.**). These results are in accordance with a peptide induced internalization of surface μ-binding sites, but not with down-regulation since the number of binding sites was not decreased in LM (**Table 4.5.**).

No significant changes either in the EC_{50} values or in the maximal stimulation by DAMGO were measured in the SPMs after these treatments. This suggests that DAMGO and its chloromethyl-keton derivative at the administered doses failed to induce detectable desensitization of the μ-opioid receptors. DAMCK but not DAMGO at 'high-dose' slightly but significantly increased the EC_{50} values in LM that might show that functional coupling to G-proteins has been attenuated (**Table 4.6.**).

It is intriguing why a peptide ligand would influence intracellular sites and not those on the cell surface. DAMGO is a highly hydrophilic ligand, thus is expected to interact only with surface receptors. However, DAMCK is more liphophilic, thus it might be able to cross the plasmamembrane.

The changes in receptor number measured by [^{3}H]DAMGO binding (**Table 4.5.**) were not always reflected in significant changes of the maximal stimulation by DAMGO in [^{35}S]GTPγS binding (**Table 4.6.**). However, it should be kept in mind that a 50% up-regulation of the receptors is expected to bring about only a 1.5-fold increase in sensitivity to the agonist (Fleming W.W.and Westfall D.P. 1988). The experimental conditions of the receptor binding and functional assays also differ that might influence the changes observed.

In conclusion, these results are in accordance with a peptide induced internalization of surface μ-binding sites, but not with down-regulation since the number of binding sites was not decreased in LM (**Table 4.5.**). DAMGO was also shown to rapidly internalize μ-opioid receptors by other investigations. Prolonged exposure to DAMGO induced desensitization and down-regulation of the μ-opioid receptors in C6 glial cells (Yabaluri and Medzihradsky, 1997). It was shown before that DAMCK interacts covalently with opioid receptors *in vitro* under appropriate conditions (Oktem et al. 1991). Although it is not known at present if it is able to alkylate the receptor *in vivo*, it displays a long-lasting analgesia that is only partly reversible by naloxone post-treatment (Szabó et al., 1999). It is however unlikely that the decrease of opioid receptor number we detected here was due to irreversible binding because a single acute injection of DAMCK did not influence the receptor binding or G-protein activation in either fraction (data not shown).

Summarizing the present results, distinct regulatory patterns of the endogenously expressed μ-opioid receptors has been shown in rat by different agonists. To our knowledge, this is the first *in vivo* demonstration of ligand-specific adaptive changes of the opioid system after chronic treatments. Such ligand-specificity of intracellular trafficking has not been observed for other G-protein coupled receptors. Thus, it raises the possibility that the unique addictive properties of opioids might be explained by their distinct pathways of receptor regulation. It will require future investigations to determine how specific features of a ligand, e.g. its chemical structure, efficacy, abuse potential and other factors are correlated with each of the molecular changes outlined here and how then tolerance and dependence are brought about.

6. Conclusions

In the present work three main features of opioid ligands, namely their structural requirements for high affinity binding, G-protein activation and regulatory mechanisms have been investigated.

In order to examine the functional aspect of ligand binding, we set up a new method, the ligand stimulated [^{35}S]GTPγS binding assay. This assay is simple to perform, and can also substitute the radioligand binding experiments. Besides determination of the potency and efficacy of the opioids, the receptor regulatory mechanisms of the opioid agonists can be studied which can complement the characterization of opioid ligands.

Binding characteristics of eight conformationally constrained analogues of dermorphins obtained by replacing D-Ala^2 with β-amino-cyclic carboxylic acids were tested to opioid receptors in rat brain crude membrane fractions.

- The analogues of dermorphins showed decreased binding activity to μ- and δ-opioid receptors.
- The attenuation in their potency was more remarkable for the μ-opioid binding.

The G-protein activation of the naturally occurring Met^5-enkephalin-Arg^6-Phe^7 and its synthetic analogues (**I:** Tyr-***D-Ala***-Gly-Phe-Met-Arg-Phe, **II:** Tyr-***D-Ala***-Gly-Phe-***D-Nle***-Arg-Phe) was examined in frog and rat brain crude membrane fractions.

- It was shown that MERF and its synthetic analogues can activate G-proteins but their ability to induce receptor stimulated guanine nucleotide exchange on the G-protein α-subunit is different.
- The substitution of Met^5 by D-Nle^5 leads to the decrease of the efficacy of analog **(II)**.
- The effect of MERF and analog **(I)** is mediated the least by μ-opioid receptors.
- The peptides we investigated showed different G-protein activation in frog and rat brain membranes.

Changes in the subcellular distribution, ligand binding parameters and G-protein coupling of μ-opioid receptors were assessed in rat brain subcellular membrane fractions after opioid treatment.

- We have shown that etorphine desensitized and internalized surface μ-opioid receptors.
- It was demonstrated by pharmacological experiments that analgesic tolerance did develop after chronic treatment of the alkaloid morphine and the highly potent opioid peptides DAMGO or DAMCK.
- We have shown the lack of the desensitization and internalization of surface μ-opioid receptors in morphine tolerant animals.
- Enhanced G-protein-receptor functional coupling was demonstrated in LM fraction of morphine tolerant rats.
- The number of the inracellular μ-opioid binding sites increased after prolonged morphine treatment.
- The opioid peptides, DAMGO and DAMCK, given chronically decreased the number of surface μ-opioid receptors with a concomitant increase in the amount of intracellular binding sites.
- Desensitization was detected neither after 'low dose' nor 'high dose' DAMGO and DAMCK treatment.
- It is concluded that chronic opioid treatment resulted in ligand specific regulation of the endogenously expressed μ-opioid receptors in rat brain. Our results suggest that internalization is not necessarily accompanied, or preceded by receptor G-protein uncoupling (desensitization) in case of μ-opioid receptors in rat brain.

Data obtained contribute to our understanding of the structure and function of opioid receptors.

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